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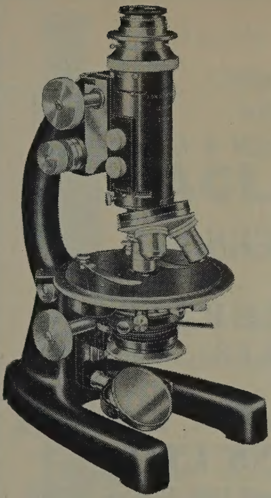

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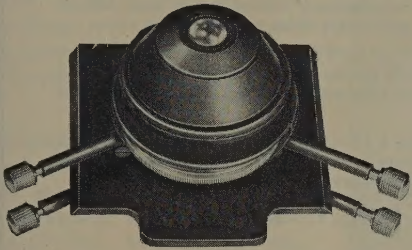

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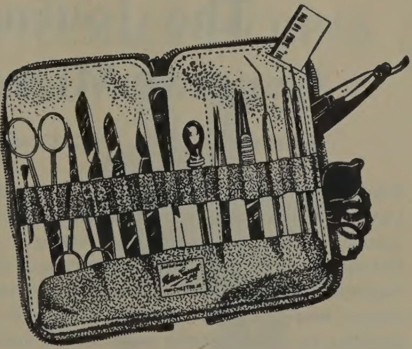

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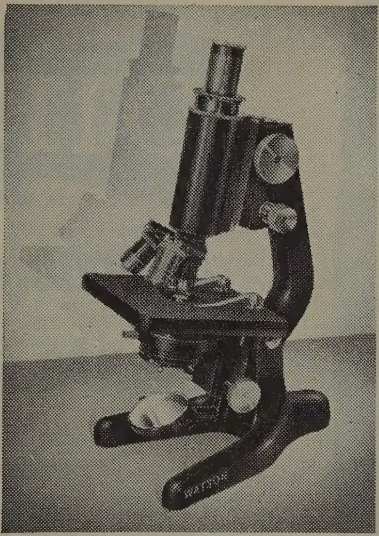

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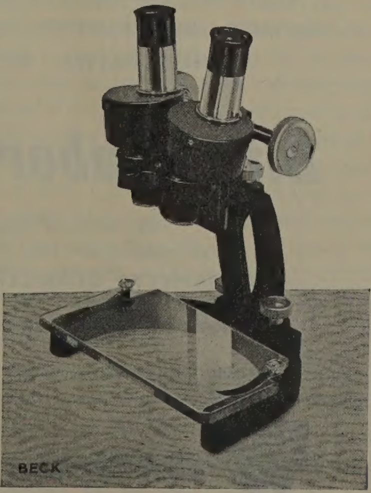

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The Small Leg-nerve of Spiders and a Probable Mechanoreceptor

By D. A. PARRY

King's College, Cambridge

(*From the Zoological Laboratory, Cambridge University*)

SUMMARY

Besides the main leg-nerve there is, in spiders, a 'small leg-nerve' which is mainly sensory. About 8 of the fibres arise from neurones which form a ganglion at the femur-patella joint and appear anatomically to be joint mechanoreceptors, but if so must supplement the lyriform organs. Cutting the small leg-nerve has no effect on the walking pattern nor on the detection of prey, which almost certainly depends on mechanoreception. The small leg-nerve contains one motor fibre which innervates the claw elevator. This muscle has a double innervation, a second fibre running in the main leg-nerve. There are interesting parallels between the small leg-nerve in spiders and *Limulus*.

INTRODUCTION

PRINGLE (1956) has recently thrown light on proprioception in chelicerates. In *Limulus* (Pringle, 1956), where 'slit' sense-organs of the campaniform type are lacking, movement at the leg-joints produces tonic and phasic responses in the 'small leg-nerve'. Pringle associates these responses with the large and small multipolar ganglion cells which occur at the joints (Stuart, 1953, reproduced in Pringle, 1956). The small leg-nerve arises from the ventral nerve-ring independently of the main leg-nerve and runs throughout the leg supplying the joints, a small area of cuticle in the tibia, and three muscles (flexor trochantaris, flexor femoris, and claw opener). In a scorpion and amblypygid Pringle (1955) showed that the slit organs (lyriform organs) characteristic of most arachnids are sensitive to strain in the cuticle like the campaniform sensillae of insects; but he also obtained a distinct large fibre response to rapid forced movement of the joints which could not be elicited by pressure on the cuticle but which appeared in response to probing at the joint membranes where there was some evidence of a sensory structure. When Dr. Pringle drew my attention to this, I found a corresponding sense organ at the femur-patella joint of the spider's leg, supplied by a small leg-nerve like the one occurring in *Limulus*. As this nerve runs close to the dorsal surface of the femur it can be cut with little damage to the leg, and operated spiders rapidly recover. It has not, however, been possible to establish the functions of either the sensory or the motor elements of the small leg-nerve by this means.

ANATOMY

Methods

Most of this work has been done on the common British house spider *Tegenaria atrica* (Koch), whose leg articulation and musculature have recently

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been described (Parry, 1957). The course of the small leg-nerve was traced by dissection under a binocular microscope, verified and amplified by serial sections which provided the micro-anatomical details. The brittleness and impermeability of the cuticle are an obstacle to good sectioning, so the following details are given of a procedure which gave adequate results. The spider is anaesthetized with carbon dioxide and the required part on the leg isolated by proximal and distal cuts. If a joint is to be examined in other than the fully flexed position it must be held at the required angle by drawing the piece of leg carefully into narrow glass tubing before fixation. Five hours in Carnoy's mixture produces adequate fixation and is followed by two baths of 95% alcohol (12 h each), one bath of methyl benzoate (12 h), one bath of 1% celloidin in methyl benzoate (24 h), one bath of benzene, and three baths (40 min each) of 56° C paraffin wax in a vacuum embedding oven. Whenever possible, recently moulted spiders are chosen. The sections were stained with Mallory's triple stain or Heidenhain's iron haematoxylin.

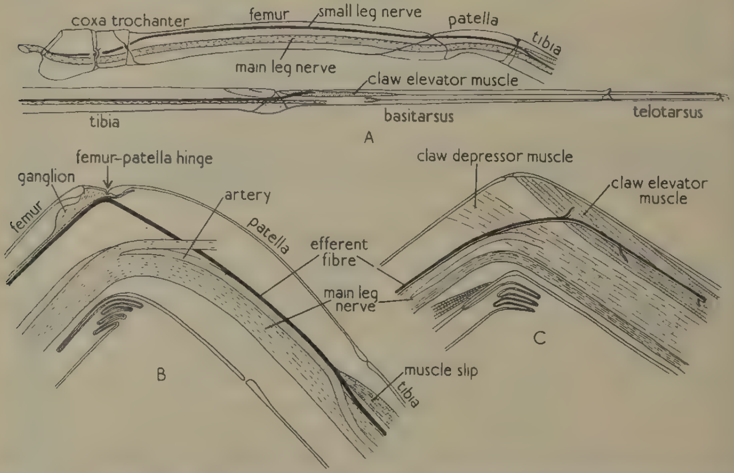

FIG. 1. A, *Tegenaria atrica*; leg showing the course of the small leg-nerve (indicated by the thick black line). B, median vertical section through the patella region showing the ganglion below the femur-patella hinge and the single efferent fibre (thick black line) with the muscle-slip attached to it. C, median vertical section through the tibia-basitarsus joint, showing the innervation of the claw-elevator muscle by the efferent fibre of the small leg-nerve and one fibre from the main leg-nerve.

The small leg-nerve

Fig. 1, A shows the course of this nerve by a line of exaggerated thickness. It branches from the main leg-nerve in the coxa, passes close to the single coxa-trochanter articulation and to the anterior trochanter-femur articulation, then runs mid-dorsally along the length of the femur to a small ganglion below the femur-patella hinge-line. Only a single axon runs beyond this point: it runs freely in the patella, lies in contact with the main leg-nerve (from which it can be freed by dissection) in the tibia, and terminates on the claw elevator muscle in the proximal region of the basitarsus. At the level of the patella-tibia articulation the fibre is closely associated with a slip of the basitarsal depressor muscle. This unusual feature is described in detail below.

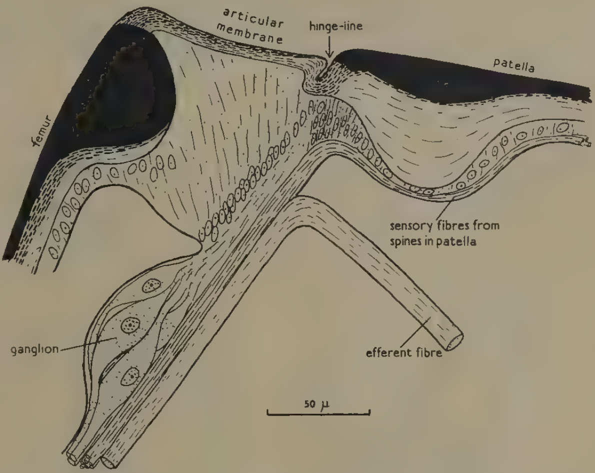

FIG. 2. *Tegenaria atrica*; median vertical section through the femur-patella hinge, showing details of the small leg-nerve and its ganglion.

The femur-patella ganglion (figs. 1, B; 2)

The patella moves on the femur about a transverse dorsal hinge-line, the two articulations being at the two ends of the hinge. The small leg-nerve runs to the mid-point of the hinge and, as stated above, only a single fibre proceeds farther down the leg. One group of small fibres penetrates the basal membrane of the patella: it has not been possible to trace these in detail, but a few fibres have been followed to sense cells at the bases of spines. The remaining fibres run to a group of 8 neurones which form a small ganglion just below and proximal to the hinge. The dendrites from these neurones can be followed running at the base of the tall epithelium which lies below the hinge, and they appear to terminate close to the cuticle at the point where most bending actually occurs.

The muscle-slip (figs. 1, A; 3)

The single fibre of the small leg-nerve which continues beyond the femur-patella hinge runs through the patella just dorsal to the main leg-nerve and the artery. At the level of the patella-tibia joint it is attached to a small group of muscle-fibres which run distally to join the more anterior of the two basitarsal flexor muscles (muscle 23, fig. 1, in Parry, 1957), most of whose fibres originate at the proximal end of the tibia. Fig. 2, A–D illustrates the relationship between the nerve-fibre and the muscle-slip. Fig. 3, A is a transverse section of the nerve-fibre in the patella just before reaching the muscle. Fig. 3, B shows the muscle-fibres arising on the nerve-fibre sheath. In fig. 3, C they have reached their full diameter but are still enclosed in the nerve-fibre sheath. In fig. 3, D the muscle-fibres are joining the basitarsal flexor muscle, whose fibres, it will be noted, are 3 or 4 times the diameter of those making up the muscle-slip. There is no indication that the nerve-fibre innervates the muscle-slip.

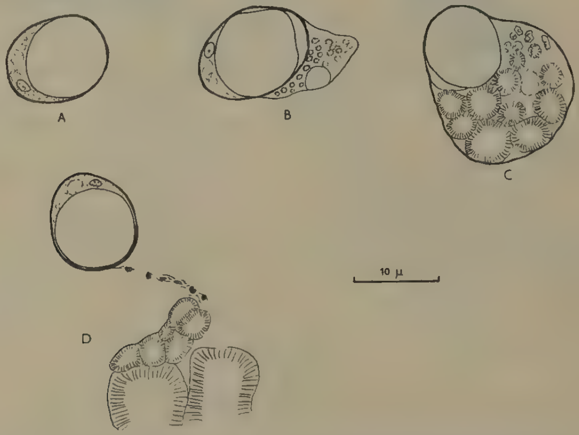

FIG. 3. *Tegenaria atrica*; A–D, successive transverse sections through the small leg-nerve (efferent fibre) at the level of the patella-tibia joint, showing the origin of the muscle-slip.

The termination of the efferent fibre

Beyond the point of origin of the muscle-slip, the small leg-nerve, composed of its single fibre, proceeds along the dorsal surface of the main leg-nerve from which it can be freed by dissection as far as the approach to the tibia-basitarsal joint. Here (fig. 1, C) it leaves the main leg-nerve in company with one other fibre. The two fibres are for a short distance loosely attached to the nerve supplying the claw depressor muscles and then proceed to the median claw elevator muscle (muscle 27, fig. 1, in Parry, 1957), to which they send repeated branches. They appear to branch simultaneously.

FUNCTION

Method. An attempt was made to establish the function of the elements of the small leg-nerve by destroying it and comparing the behaviour of operated spiders and normal ones. The nerve can be cut in the femur where it runs close to the dorsal surface between the paired patella flexor muscles. The spider is covered with an inverted Perspex box which fits closely to the sides of the body, allowing the legs to protrude. Carbon dioxide is passed through the box. The chosen leg is clamped with light pressure at the base of the femur to prevent loss of blood and a flap of cuticle is cut near the distal end. This is folded back and the small leg-nerve destroyed with a hooked needle.

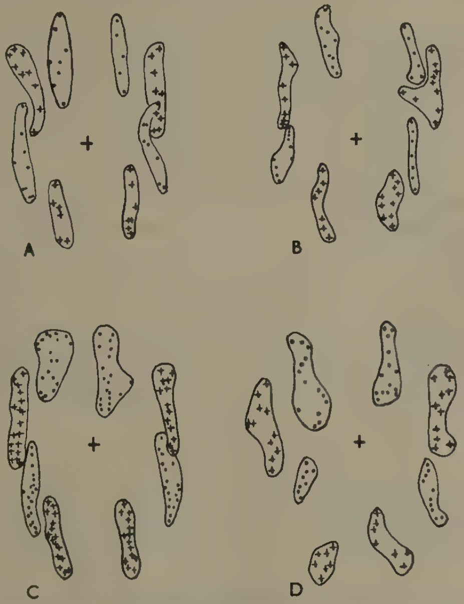

FIG. 4. *Tegenaria atrica*. Walking patterns of normal spiders (A, B); and of spiders in which the small leg-nerve of the first left leg has been cut (C, D).

The flap is then replaced and sealed with paraffin wax. Sometimes the operated leg is used as soon as the spider recovers from the anaesthetic, while at other times it is held stiffly for some hours—probably when the clamp has been too tight.

Three aspects of behaviour were examined, namely, claw retraction, walking, and prey detection. As the results were all negative the experiments will be described briefly.

Claw retraction. The spider is held down by means of the box mentioned above, no anaesthetic being used, and the legs touched with a needle so that the claws are set in motion. The activity of three claws on operated legs (two different spiders) appeared normal.

Walking. During the course of this work many spiders with one or more small leg-nerves cut were watched as they walked, both on a flat surface and on their webs, and no abnormality could be detected. To confirm this impression spiders with one operated leg were filmed while walking and the 'stepping pattern' of their legs compared. The 'stepping pattern' is a diagram showing the position of each foot (relative to the body) in successive frames of the film during a few cycles of movement. Typical patterns are shown in fig. 4: A and B were taken before, and C and D after the front left leg-nerve had been cut, and it can be seen that the operation had no significant effect.

Detection of prey in the web. Spiders kept in crystallizing bowls of 9-in. diameter build webs across the bowl. A strip of blotting paper round the inside circumference, near the top, helps the spider to get a footing and usually determines the plane of the web. The spider constructs a retreat on the edge from which it will usually emerge immediately a small cockroach is dropped anywhere on the web, and run straight to the prey. This response will take place in darkness and there is little doubt that it depends on a mechanical sense of considerable delicacy.

To test the role of the small leg-nerve in this response, the nerve was cut in each leg, usually over several days. A cockroach was then dropped on the web every day or so, and the response recorded. A negative response in such an experiment does not, of course, necessarily mean that the prey is not detected: normal spiders do not invariably respond. But a *positive* response from an operated spider indicates that prey detection is not dependent upon any element of the small leg-nerve. That this is the case is indicated by the following results.

Spider	*Date of operation*	*Dates with response*	*Dates with no response*
A	17 Aug.: all legs and palps	18, 20, 21, 22, 23, 26, 28, 30 Aug.; 11 Sept.	24, 29 Aug.; 10 Sept.
B	8 Aug.: all legs	13, 14, 18, 21, 23, 24, 27 Aug.	20, 22, 29, 30, 31 Aug.; 1, 11, 12 Sept.
C	16 Aug.: all legs	18, 20, 22, 23, 24, 27, 30, 31 Aug.; 10 Sept.	21, 28, 29 Aug.
D	1 Oct.: all legs; 9 Oct. palps	6, 9, 10 Oct.	2, 3, 4 Oct.

The date of operation is that on which the last leg-nerve was cut. It is not known whether a 'small leg-nerve' occurs in the palps, but in two experiments these were removed.

Notes on subsequent examination of operated spiders

Spiders A, C, and D: all legs dissected and in each one the small leg-nerve was found broken or was missing in the femur.

Spider B: all legs sectioned and the efferent fibre in the patella either found to have the dark appearance characteristic of a cut nerve or else to be missing. The cut in the femur could not always be confirmed as the nerve sometimes extended to the confused region of the operation.

These results show that no elements of the small leg-nerve, nor any sensory terminations in the palps, are essential for the detection of prey in the web.

DISCUSSION

I have shown that the small leg-nerve in the spider is mainly sensory, containing about 8 axons from a ganglion situated below the femur-patella hinge and a number of axons from sensory hairs on the patella. The single motor axon innervates the claw retractor muscle which also receives another axon from the main leg-nerve. The following points call for comment.

(*a*) There is an interesting parallel between the presence of a small leg-nerve in spiders (and probably in scorpions—Pringle, private communication) and in *Limulus* (Patten & Redenbaugh, 1900; Pringle, 1956). In the latter, also, it is mainly sensory, supplying groups of multipolar nerve-cells at four of the joints and also a small area of sensory hairs on the tibia. Its motor axons innervate three muscles: the claw elevator (opener) and the flexors of the trochanter and femur.

(*b*) *Limulus* has no cuticular mechanoreceptors of the lyriform-campaniform type and Pringle (1956) has shown that the multipolar nerve-cells supplied by the small leg-nerve are proprioceptive in function. He suggests that the smaller cells, lying close to the joint membrane, are non-adapting position receptors; while the larger cells, lying some distance proximal to each joint, are phasic movement receptors. On analogy with *Limulus* it is tempting to suggest that the femur-patella ganglion in the spider leg is also proprioceptive, but if so it must in some way supplement the lyriform organs which are present at all the leg joints and which Pringle (1955) has shown to be mechanoreceptors. Of alternative functions the detection of prey seemed a likely possibility—it almost certainly depends on an extremely delicate mechanical sense—but this idea has not been borne out by the experiments described above. The problem should be quickly solved when it becomes possible to record electro-physiologically from spider nerves.

(*c*) There is, however, a further parallel between the *Limulus* and spider small leg-nerves which suggests that the latter may be concerned in some way with mechanoreception. In *Limulus* Pringle points out that the small leg-nerve is 'always rather accurately located between points at the femuro-tibial and trochantero-femoral joints which do not move relative to one another when the segments flex and extend; [while,] at the trochantero-femoral joint, a small, fine muscle with close striation . . . runs from trochanter to femur and is attached by connective tissue to the small nerve where this runs round the inner curve of the trochanter. It is hard to see that this muscle can have any function other than to hold the nerve in this region away from the cuticle of the trochanter at or very near to the position of zero stretch when this segment moves. Such an arrangement is well suited to provide a fixed reference line for sensory structures responding to movement through the pull of other connective tissue strands whose outer insertion is on moving parts.' In the spider the small leg-nerve passes very close to the coxa-trochanter articulation, the anterior trochanter-femur articulation, and the femur-patella hinge line, so that movement at these joints will have little or no effect on the nerve. In the patella itself the nerve (now consisting of a single fibre) runs freely in the haemocoel to become loosely attached to the main leg-nerve in the tibia, and it is at the level of the patella-tibia joint that the small slip of muscle is attached to the nerve. This muscle could, like the muscle in *Limulus* described by Pringle, regulate tension in the nerve and at the femur-patella ganglion.

(*d*) The efferent innervation of the claw elevator appears to consist of two fibres only—one from the small leg-nerve and one from the main leg-nerve. A double innervation—by a fast and a slow fibre—is the common condition in insects, and Hoyle (1957) has recently shown that the claw-closer muscle in *Limulus* also receives a fast and slow fibre. If the spider's small leg-nerve carries the slow fibre to the claw elevator, this would probably explain why cutting the nerve had no apparent effect on the response of the claw to general stimulation.

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An Anatomical, Histological, and Histochemical Study of the Gut of the Brachiopod, *Crania anomala*

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With one plate (fig. 2)

SUMMARY

The gut of *Crania anomala* has been studied morphologically and histochemically. It is attached to the body-wall by dorsal and ventral mesenteries with the exception of the posterior part of the intestine, which lies free in the right half of the visceral cavity. The gut-wall consists of an inner columnar epithelium, a connective-tissue stroma, and an investing squamous mesothelium. The columnar epithelium comprises ordinary epithelial cells, some goblet cells, and occasional phagocytes.

The cytoplasmic inclusions of the gut epithelium include pigment granules, glycogen granules, lipochondria, and goblet-cell globules. The lipochondria contain a phospholipid. The goblet-cell globules contain a muco- or glycoprotein, and are extruded into the lumen of the gut presumably for lubrication and for the entanglement of food particles. Extranuclear DNA, presumably originating from the nucleus, occurs in the cytoplasm of the ordinary epithelial cells in the digestive diverticula.

THE anatomy of the gut in *Crania anomala* Müller was described by Joubin (1886) and Blochmann (1892), both of whom also made some observations on its histology. The present paper gives an account of the structure of the gut and the results of some histochemical tests.

MATERIAL AND METHODS

Specimens of *C. anomala* were dredged off Little Cumbrae, Scotland. The adults were removed from the stones to which they were attached, and fixed at once at the Marine Station, Millport. Histochemical tests were carried out in the Cytological Laboratory, University of Oxford. Details of these tests are given in the appendix (table 1).

RESULTS

Anatomy of the gut

The gut in *C. anomala* is longer than the visceral cavity. The mouth is a horizontal slit situated in the sagittal plane where the two brachial grooves meet, as Joubin (1886) and Blochmann (1892) noted. The pharynx (fig. 1, A), into which the mouth opens, is a dorsally convex curved cylinder embedded in the bases of the brachia. Sinuses communicating with the visceral cavity abound in the pharyngeal connective-tissue envelope. The gut leaves the bases of the brachia as a short straight oesophagus (fig. 1, A) of uniform diameter. This lies medially in the anterior part of the visceral cavity and is

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surrounded by a thin sheath of compact connective tissue. The stomach (fig. 1, A) increases in diameter from the oesophageal end, near which it receives antero-dorsally the ducts of the two digestive diverticula. The stomach reaches its greatest diameter just in front of a marked constriction. Posteriorly it narrows and eventually becomes a tube of uniform diameter. This narrow tube curves ventrally towards the left. A slight constriction near the end of the curved region indicates the position of a sphincter (fig. 1, A–D), which marks the beginning of the intestine.

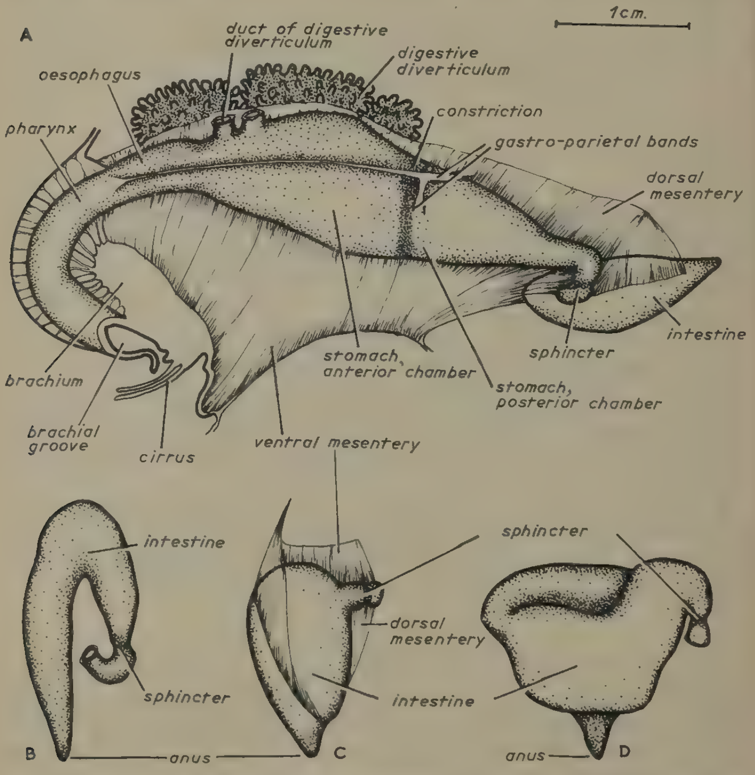

FIG. 1. The gut of *C. anomala*. A, lateral view of the gut from an adult with a dorsal valve 14 mm in transverse diameter. B, ventral view of a contracted intestine. Mesenteries omitted. C, ventral view of a moderately distended intestine. D, ventral view of a fully distended intestine. Mesenteries omitted.

The intestine is V-shaped with its vertex directed anteriorly (fig. 1, B). From the sphincter it increases in diameter only to taper again near the anus. When distended, it assumes grotesque shapes as a result of unequal dilation of its various regions, and its anterior portion then tends to be directed transversely to the longitudinal axis of the animal (fig. 1, D). The anus (fig. 1, B–D) opens into the mantle cavity posteriorly, as Joubin (1886) first noted. The last part of the intestine lies in a conical posterior extension of the visceral cavity. This conical extension lies to the right of, and behind, the place of attachment of the dorsal and ventral mesenteries to the intestine. Hence the last part of the intestine lies in the right half of the visceral cavity, as in other inarticulates.

There are two digestive diverticula separated by a median dorsal mesentery. The right one arises from the antero-dorsal surface of the anterior chamber of the stomach to the right of the dorsal mesentery by a wide duct, which immediately branches into two to serve the two constituent lobes of the diverticulum—an anterior and a posterior lobe. The anterior lobe (Joubin's superior and middle lobes) usually consists of 7 lobules of different sizes. The posterior lobe (Joubin's inferior lobe) comprises 6 lobules. The left diverticulum arises from a corresponding position to the left of the dorsal mesentery usually by two ducts which lie in an antero-posterior direction (fig. 1, A). The anterior duct serves the anterior lobe (Joubin's superior and middle lobes) of 9 lobules; the other serves the posterior lobe (Joubin's inferior lobe) of 8 lobules. Each lobule is made up of a number of small branched ductules and acini. The lobulation is not regular, as in many specimens there are some isolated branched ductules and acini arising near the bases of the big ducts. Moreover, lobulation of the more ventral part of each lobe is variable.

A dorsal mesentery (fig. 1, A) extends from the mid-dorsal surface of the gut to the dorsal body-wall. A ventral mesentery (fig. 1, A) descends mid-ventrally from the pharynx, oesophagus, and stomach to the bases of the brachia and the ventral body-wall, but along the curved part of the intestine it extends antero-ventrally to the ventral body-wall. The dorsal and ventral mesenteries divide the visceral cavity into two compartments and terminate some distance from the anus. Since the line of attachment of the ventral mesentery along the anterior part of the intestine is directed laterally towards the right, the greater part of the originally left lateral side of the intestine faces ventrally instead. This presumably is caused by asymmetrical growth of the anterior part of the intestine during development, which produces a clockwise rotation through about 90° when viewed from the anterior end.

A low longitudinal ridge of connective tissue, which may be called the longitudinal gastro-parietal band (Blochmann's ileo-parietal band), extends along each side of the oesophagus and stomach. Near the constriction between the two chambers of the stomach the band, continuing in the same direction, leaves the surface of the stomach. It crosses and overlies a narrow transverse band of connective tissue, which may be called the transverse gastro-parietal band (gastro-parietal band of Blochmann (1900)), that extends laterally from the mid-dorsal region of the stomach just behind the constriction. On each side the longitudinal band fuses with the transverse gastro-parietal. About 1 mm from this point of fusion the former expands posteriorly into a large triangular piece of transparent membrane, whose lateral edge becomes attached to the lateral body-wall and whose lower surface suspends the gonad. The other band continues ventrally forward on each side to support the nephridium.

Histology of the gut

The gut is lined internally by a simple columnar epithelium resting on a basement membrane, which forms the innermost layer of the connective-tissue stroma. In the free part of the gut this is invested with squamous mesothelium, in many regions of which cilia have been observed, as Blochmann (1892) previously noted. Joubin (1886) mentioned 'la gaine de cartilage', which corresponds to either the basement membrane or the connective-tissue stroma of this paper.

The inner epithelium of the gut shows circular and longitudinal folds in the pharynx, but only longitudinal ones in the oesophagus. Between this and the stomach is a circular fold where the nuclei on the oesophageal side are slender and long as in the oesophageal epithelium, while those on the stomach side are shorter and stouter as in the stomach epithelium. Two parallel grooves run transversely across the dorsal and lateral walls of the anterior chamber of the stomach, approximately equidistant from the openings of the digestive diverticula and the gastric constriction. The epithelium that intervenes between these grooves has the form of a fold, which reminds one of a similar but shorter curved fold that runs along the right wall of the anterior chamber of the stomach in *Lingula* (Chuang, 1959, fig. 8, *a*). A ciliated epithelial groove runs longitudinally along the floor of the anterior chamber of the stomach and continues postero-dorsally from the floor to the roof of the posterior chamber by way of the right lateral wall (Chuang, 1959). The intestinal epithelium bears some circular folds, especially when the intestine is not fully distended.

The columnar epithelium is ciliated everywhere except in the acini of the digestive diverticula, and consists of ordinary epithelial cells interspersed with goblet cells and occasional wandering phagocytes. In the pharynx and the oesophagus the ordinary epithelial cells are tall and slender, with elongated nuclei (fig. 2, A). Shorter cells with shorter, broader nuclei line the stomach and the intestine. In the stomach these cells have a distinct border formed of

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Fig. 2 (plate). The gut of *C. anomala*.

A, sagittal section through the basal region of the roof epithelium of the pharynx. Zenker fixation; Feulgen test for DNA.

B, transverse section through part of an acinus of a digestive diverticulum. Zenker fixation; Feulgen test.

C, longitudinal section through part of a duct of a digestive diverticulum. Zenker fixation; periodic acid / Schiff technique.

D, sagittal section through the basal region of the floor epithelium of the pharynx. Weak Bouin fixation; Baker's pyridine extraction test.

E, transverse section through part of an acinus of a digestive diverticulum. Fixation and technique as D. Note the strongly positive reaction of the muscle strands.

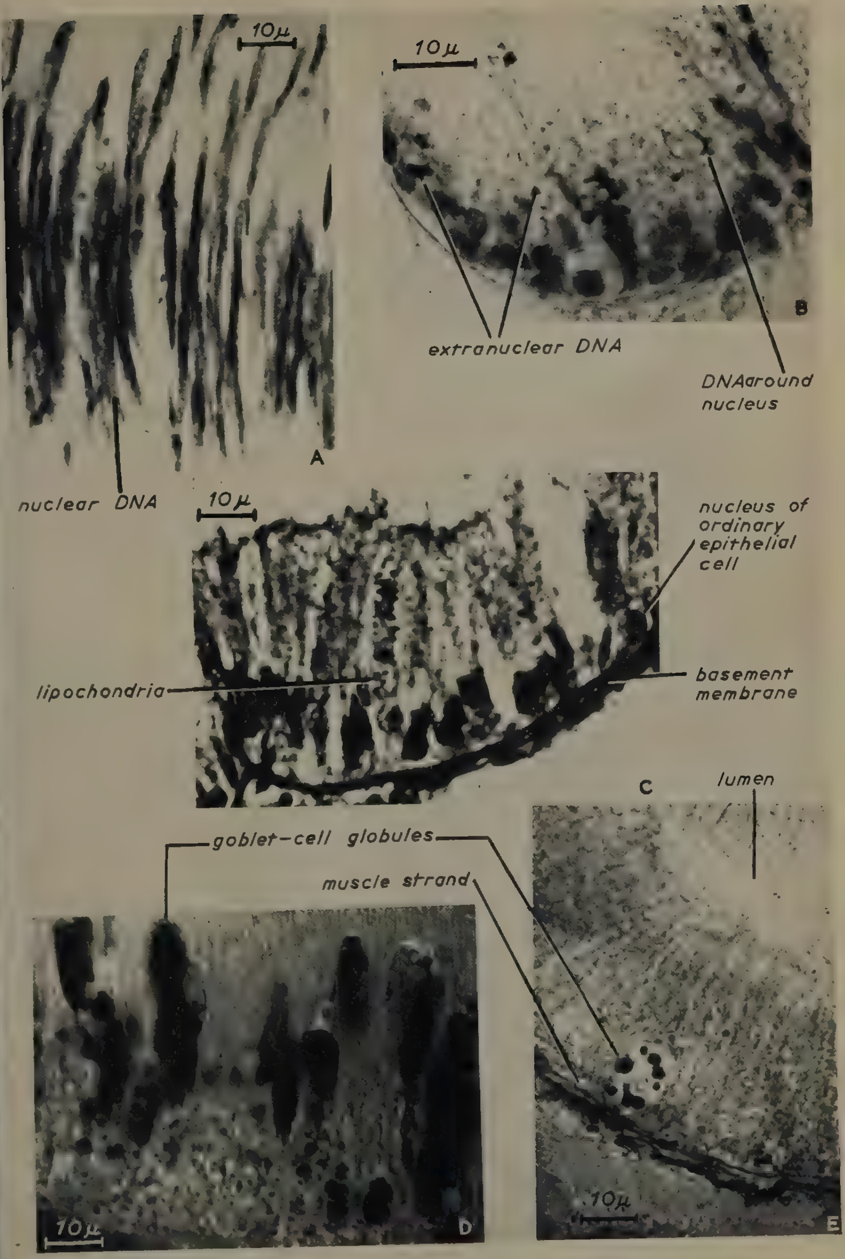

FIG. 2

S. H. CHUANG

the prominent roots of strong cilia. This border is widest and most prominent in the funnel-shaped region of the posterior chamber of the stomach, becoming narrower and less conspicuous both anteriorly and posteriorly. Short columnar cells with subspherical nuclei occur in the digestive diverticula (fig. 2, B, C), but whereas the distal ends of the cells in the ducts are flat, those in the acini bulge out in varying degrees to form an uneven surface. Blobs of cytoplasm break away from the domes of these acinar cells to appear in the lumen, as in *Lingula unguis* (Chuang, 1959).

The goblet cells are especially abundant in the stomach, intestine, and floor of the pharynx (fig. 2, D), but comparatively scarce in the digestive diverticula (fig. 2, E) and the oesophagus. They usually lie with their distended part between the bases of neighbouring cells. Those with few small globules tend to have large subspherical nuclei, while those with numerous large globules have small flattened nuclei.

In the connective-tissue stroma are found a basement membrane on the inner epithelial side, a network of connective-tissue fibres and cells in the middle, and a network of separate muscle strands externally. The connective-tissue fibres are fine, single, branching fibres. The muscular network consists of an inner layer of broad strands arranged circularly and an outer layer of narrow strands arranged longitudinally along the gut, with the former predominating. This muscular network is especially well developed in the pharynx, where the circular strands are most conspicuous. The digestive diverticula, though less muscular than the pharynx, are as muscular as the stomach. Presumably owing to dilation, the intestine appears the least muscular part of the alimentary canal, especially in its anterior region.

Of the cytoplasmic inclusions in the gut-wall only the pigment granules and the subspherical globules are easily recognizable under the light microscope. These pigment granules occur in the epithelium, connective-tissue cells, mesothelium, and lumen of both the digestive diverticula and the stomach. They are irregularly shaped inclusions in various shades of yellow, about 2 μ in diameter. The subspherical globules vary in diameter from less than 1 μ to about 12 μ and can conveniently be divided into two groups. One group of globules, henceforth called lipochondria, varies from less than 1 μ to about 3 μ in diameter. They occur in the ordinary epithelial cells throughout the gut, in the detached blobs of the acinar cells in the digestive diverticula, and in the connective-tissue cells and mesothelium. The other group comprises the globules confined to the goblet cells (fig. 2, D, E); these measure from less than 1 μ to 12 μ in diameter. Food vacuoles have been observed to occur singly in the distal part of the acinar epithelium.

Histochemistry of the gut

Amino-acids and proteins. The nucleus and cytoplasm of all the cells of the gut were weakly positive to the Hg/nitrite test for phenols (Baker, 1956), and the modified Sakaguchi test for arginine (Baker, 1947). They therefore contain some tyrosine and arginine. The tests also indicate the presence of tyrosine and arginine in the ground substance, fibres and basement membrane of the connective tissue in the gut.

The globules in the goblet cells gave a positive reaction to the acid haematein test after pyridine extraction (fig. 2, D, E), the Hg/nitrite test for phenols, and the modified Sakaguchi test for arginine. They also gave a positive reaction to PAS, which was not affected by amylase. They showed no metachromasy with toluidine blue. Hence the protein in these globules should be regarded as a muco- or glycoprotein (Pearse, 1953).

Nucleic acids. Fine, irregular granules in the nuclei of cells (fig. 2, A, B) were positive to the Feulgen test for DNA (Feulgen and Rossenbeck, 1924). These DNA granules were sparse in the nuclei of the intestinal epithelium, denser but variable in the epithelial nuclei of other regions of the gut, and densest in the nuclei of the mesothelial and connective-tissue cells.

Similar granules positive to the Feulgen test were found in the cytoplasm of the epithelial cells in the digestive diverticula. These extranuclear DNA granules had a wider range of sizes than the intranuclear ones. Many of them occurred in the neighbourhood of nuclei deficient in DNA (fig. 2, B), although a few were found in the distal part of the cytoplasm. This therefore provides evidence for the belief that these cytoplasmic DNA granules originated from the nuclei and that they migrated towards the lumen.

Carbohydrates. The weakly positive reaction to PAS of some extremely minute granules in the distal part of the gut epithelium from the pharynx to the stomach, including the digestive diverticula, was abolished by previous incubation of the slide with saliva as control. These granules therefore contain a carbohydrate soluble by amylase, presumably glycogen.

The connective tissue of the gut gave a positive reaction to both the Hg/nitrite test for phenols and the modified Sakaguchi test for arginine. The basement membrane and the fibres most probably contain an absorbed acid mucopolysaccharide, because of their strong γ-metachromasy with toluidine blue and their amylase-fast positive reaction to PAS. After treatment with chromic acid or sulphuric acid, the γ-metachromasy of the basement membrane and the connective-tissue ground substance was increased; these structures therefore presumably also contain some neutral mucopolysaccharide.

Lipids. A negative reaction to the standard tests for lipids was given by the ground cytoplasm of the gut cells. The lipochondria of the epithelial cells were positive to Sudan IV, Sudan black, and the acid haematein test (Baker, 1946). These lipochondria contain a phospholipid, since their positive reaction to the acid haematein test is abolished by previous pyridine extraction.

DISCUSSION

The gut of *C. anomala* does not differ much from the gut of other recent inarticulate brachiopods. In this paper the different regions of the gut are named by homology with those in *Lingula* (Chuang, 1959). The relationship to the terms used by Joubin (1886) and Blochmann (1892) is summarized in table 2 of the appendix.

Although the anus opens posteriorly in the median line—a character that Blochmann (1892) regarded as primitive—a short posterior part of the intestine, situated to the right of both the dorsal and the ventral mesenteries, actually lies in the right half of the visceral cavity, as in other recent inarticulates. The stomach, unlike that in *Lingula*, does not reach the posterior end of the visceral cavity, nor does it show as much morphological difference between its two chambers as that of *Lingula*. The greater part of the posterior chamber of the stomach and the short, distended intestine are of comparatively larger diameter than the corresponding regions in *Lingula*. Presumably it was necessary for *C. anomala* to make good the reduction in capacity through a decrease in length by an increase in diameter of some regions of the gut. Presumably all these and the characteristic, posterior position of the anus may be an adaptation necessitated by the greatly reduced visceral cavity.

The glycogen reported in the epithelium of the gut from the pharynx to the stomach, including the digestive diverticula, is a reserve food store, synthesized from food carbohydrate, as Chuang (1959) has reported the presence of an amylase acting on carbohydrate in *Lingula*. The globules of muco- or glycoprotein produced by the goblet cells are extruded into the lumen of the gut presumably for the lubrication of the epithelium and the entanglement of food particles.

The inarticulate gut is a difficult tissue for the demonstration of muscle strands, as Chuang (1959) managed to demonstrate the presence of the circular and longitudinal strands only after trying several staining methods. Thanks to Baker's acid haematein test following pyridine extraction (1946), which stains the proteins of the muscle strands but leaves unstained the basement membrane, fibres, and ground substance of the connective-tissue sheath, it is possible to demonstrate them in the digestive diverticula of *C. anomala* (fig. 2, E). Blochmann (1892) remarked, with reference to these fibres, 'Bei den Leberläppchen selbst konnte ich keine Muskelfasern mehr nachweisen.' The entire gut-wall has been found to possess both circular and longitudinal muscle strands. The circular strands are most prominent in the relaxed pharynx and oesophagus, where the broad circular strands form distinct sheets, whereas the thin longitudinal strands remain separated from one another. Blochmann's failure to find one set of muscle strands or the other was presumably due to the different degrees of contraction of the various regions of the gut.

There is evidence of intracellular digestion in the digestive diverticula, since numerous food vacuoles were observed in the distal part of the acinar epithelium in *C. anomala*. The pulsation cycles, which are responsible for the transport of suspended particles between the stomach and the digestive diverticula in *Lingula* (Chuang, 1959), could very well occur in *Crania*, since the wall of the digestive diverticula in *Crania* appears more muscular than that in *Lingula*.

I wish to thank Dr. J. R. Baker, F.R.S., under whose supervision this work was carried out, for the help I have received and the laboratory facilities enjoyed. My thanks are due also to Professor Sir A. C. Hardy, F.R.S., for facilities in his department, to Mrs. B. M. Jordan-Luke and Dr. J. T. Y. Chou for many helpful suggestions, and to Dr. A. P. Orr, Deputy Director, and the staff of the Marine Station, Millport, for the supply of the specimens, and especially Mr. E. Latham for fixing some of them. The work was carried out during study leave granted by the University of Malaya.

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TABLE I

A summary of the histochemistry of the gut of Crania anomala

Practical notes					*Results*				
Test or technique	*Fixation*	*Embedding medium*	*Section thickness in μ*	*Reference*	*Lipochondria*	*Goblet cell globule*	*Connective-tissue, fibre*	*Connective-tissue, basement membrane*	*Connective-tissue, ground substance*
Hg/nitrite	FS	C	15	Baker, 1956	++	+++	++	++	++
Sakaguchi	Z	P	6	Baker, 1947	o	+	++	+	+
Feulgen	Z	P	6	Feulgen and Rossenbeck, 1924	o	o	o	o	o
Feulgen: no hydrolysis	Z	P	6	Feulgen and Rossenbeck, 1924	o	o	o	o	o
PAS	Z	P	6	McManus, 1948	+	++	++	++	+
PAS after saliva	Z	P	6	Pearse, 1954	+	++	++	++	+
Toluidine blue for metachromasy	Z	P	6	Baker, unpublished	++β	o	+++γ	++γ	+γ
Toluidine blue after chromic acid	Z	P	6	Clara, 1940	o	+++γ	+++γ	+++γ	++γ
Toluidine blue after sulphuric acid	Z	P	6	Lison, 1953	o	+++γ	+++γ	+++γ	++γ
Basic fuchsine for basiphilia	Z	P	6	..	++	+++	—	++	—
Acid haematein	FCa+PC	G	10	Baker, 1946	+	o	o	o	o
Acid haematein: pyridine extraction	WB+PE	G	10	Baker, 1946	o	++	o	o	o
Sudan IV	FCa+PC	G	10	Herxheimer, 1901	+	o	o	o	o
Sudan black	FCa+PC	G	10	Baker, 1944, 1949	++	o	o	o	o
Nile blue	FCa+PC	G	10	Cain, 1947, 1948	o	o	o	o	o
Gomori for alkaline phosphatase	ALC/AC	P	8	Gomori, 1952	o	o	o	o	o
Cobalt method for calcium	F/ALC	P	8	..	o	o	o	o	o

KEY: ALC/AC = fixation in absolute alcohol / acetone mixture; C = collodion; F/ALC = formaldehyde / absolute alcohol mixture; FCa+PC = formaldehyde calcium with postchroming; FS = formaldehyde saline; G = gelatine; P = paraffin; WB+PE = weak Bouin followed by pyridine extraction; Z = Zenker's fluid; β = beta metachromasy; γ = gamma metachromasy.

TABLE 2

Division of the gut in Crania anomala *according to various authors*

Author	*Gut*					
Chuang	pharynx	oesophagus	anterior chamber of the stomach	digestive diverticulum	posterior chamber of the stomach	intestine
Blochmann	oesophagus	oesophagus	stomach	liver	stomach and small intestine	hindgut
Joubin	oesophagus	oesophagus	anterior and middle parts of stomach	liver	posterior part of stomach and intestine	rectum

Further Observations on the Development of the Peptic Cells of the Rabbit's Stomach

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SUMMARY

1. The loss of the mucinogen component of the peptic granules begins during the fourth week of post-natal life (400 g) at a site on or near the greater curvature of the stomach adjacent to the pyloric antrum. It reaches the lesser curvature at about the sixth week (750 g).

2. The loss progresses slowly across the ventral and dorsal walls of the stomach and more rapidly along the greater curvature.

3. At 11–12 weeks (1,800 g) only a few peptic cells on the greater curvature and adjacent to the oesophageal opening contain PAS-positive granules.

4. Except for a few granules in an occasional peptic cell, no mucinogen can be demonstrated in these cells after the fourteenth week (1,900 g).

5. The stage seems to be correlated more closely with the weight than with the age of the animals.

INTRODUCTION

IT has recently been shown (Menzies, 1958) that during the early weeks of post-natal life all the granules in the peptic cells of the rabbit's stomach contain a mucinogen component. This component, as shown by the PAS reaction, is lost, first in the cells at the base of the glands and then up the tubules towards the crypts. As all the samples of the gastric mucosa were taken from a similar situation on the greater curvature of the stomach, where it was shown that the loss occurs between the fourth and sixth weeks of post-natal life, it seemed desirable to ascertain if this loss occurs in all areas of the stomach at the same time, and that is the purpose of the present investigation.

MATERIALS AND METHODS

The stomachs from three adult rabbits were used to find the exact areas that contained peptic cells. Fifty-six young animals were used, from 10 litters. These were killed and weighed at intervals from 2 to 16 weeks after birth. Not less than 4 animals were examined at each interval from the third to the eleventh week inclusive. In order to try to prevent an excessive loss of peptic granules through feeding, all animals over 4 weeks old were starved for a period of 12 h before being killed. During this period of starvation they were kept in separate cages and allowed fluid to drink as much as they wished.

As it has been suggested that pyronin increases protein synthesis (Hoffman, 1952), two other litters of rabbits were examined that had been fed with 1% pyronin (Gurr's G). This was placed in the drinking fluid of the mothers from the time of conception, and in the drinking fluid of the young rabbits from these litters after the time of weaning (5 weeks).

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Histochemical and cytological techniques

In order to construct a map of the sites of the peptic cells that contained either (*a*) PAS-positive granules, or (*b*) granules that had lost their PAS-positive reaction (Menzies, 1958), from 12 to 26 representative pieces of the stomach wall were removed and examined histologically. The reader is referred to fig. 1, which shows the sites of the pieces of the mucosa examined.

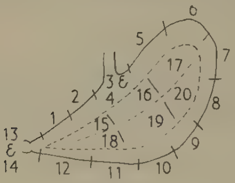

Fig. 1

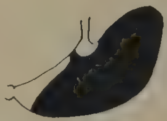

Fig. 2

Fig. 1. Diagram of the ventral aspect of the stomach to show the site and extent of the pieces of mucosa taken.

Fig. 2. Diagram showing the distribution of the peptic cells (black), in relation to the greater and lesser curvatures and the ventral or dorsal walls of the adult rabbit's stomach. This diagram could equally well represent any typical rabbit weighing more than 200 g (i.e. at least 3 weeks old). Note the relatively small area occupied by peptic cells on the lesser curvature.

Nos. 1 and 2 were taken from the ventral and dorsal walls of the stomach to include the lesser curvature from the pylorus to the oesophagus. Nos. 5 to 12 include the whole of the greater curvature and adjacent stomach wall. Nos. 3 and 4 include both sides of the oesophageal inlet and the adjacent portions of greater and lesser curvature of the stomach. Nos. 13 and 14 include both sides of the stomach adjacent to the pyloric sphincter. The remaining two pieces left were the ventral and dorsal walls; these were each cut into about 6 pieces and numbered. Nos. 15 to 20 represent the ventral wall of the stomach. Nos. 21 to 26, not shown in the diagram, represent the dorsal wall. It was found that the number of pieces necessary to cover the above areas could be reduced to 12 in animals under 5 weeks old. The pieces of mucous membrane were pinned out on cork, orientated, numbered, fixed for Bowie's method (Bowie, 1936), and subjected to the following procedures.

A. For the mucinogen component: the periodic acid / Schiff technique.

B. For the pepsinogen component: Bowie's method.

After appropriate fixation all pepsinogen granules at all stages colour blue with Bowie's method, whether they contain a mucinogen component or not, whereas only those containing a mucinogen component colour with the periodic acid / Schiff technique.

RESULTS

Peptic cells in adult rabbits

As has been reported in other species, the peptic cells in the rabbit are found (fig. 2) only in the body (or fundus) of the stomach, being absent from the pyloric area and a small region around the oesophagus (both these areas being white in fig. 2). Peptic cells occupy only a very small area on the lesser curvature (figs. 2 and 3), an area much smaller than any I have seen described in the literature for other mammals; but they approach much nearer to the pyloric sphincter on the greater curvature (see fig. 2). On the ventral (fig. 2) and dorsal walls of the stomach the limit of the area occupied by peptic cells is represented by a line gently curved towards the pylorus from greater to lesser curvature. Except in a small transitional area adjoining this line, the peptic cells are found in the bases and bodies of the gastric tubules. As this line is approached they are seen to be absent first from the upper regions of the bodies and later from the lower portions of the bodies, being thus present only in the bases of the tubules, until finally they completely disappear rather abruptly.

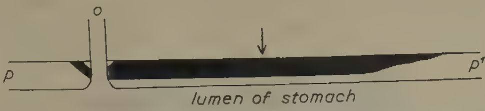

FIG. 3. Diagram of the distribution of peptic cells (black) along the greater and lesser curvatures of the adult rabbit's stomach. *o*, oesophagus; *p*, the pyloric part of the stomach on the lesser curvature; *p′*, the pyloric part of the stomach on the greater curvature. The arrow is at the site on the greater curvature opposite the opening of the oesophagus.

Peptic cells in young rabbits

Animals 3 weeks old. The distribution of peptic cells at this age is similar to that of the adult (see fig. 2), but whereas in the latter they are found in the lower two-thirds of the gastric tubules, at 3 weeks old they show an uneven distribution in all cases, being in many areas absent from the bases of the tubules but in others extending up to the crypts. The majority, however, are found in the bases and bodies of the tubules. This irregular distribution of peptic cells within the gastric tubules seems to bear no relation to the position of the tubules in the stomach wall. One animal (examined at 2 weeks old) showed peptic cells full of Bowie-positive granules, occupying three-quarters of the length of the lesser curvature. This occurrence, which according to Hirschowitz (1958) may be a seasonal phenomenon, was not seen in any of the other animals.

All the peptic granules examined in animals 3 weeks old are PAS-positive (fig. 4, A).

Animals 4 weeks old. The results with one animal (of 260 g) could not be distinguished from those of the preceding group; but the others (8 animals, each weighing between 350 and 480 g) show a marked increase in the number of peptic cells present, and a distribution in the tubules very similar to that in the adult.

Except for a small area on the lesser curvature of the stomach and the adjacent portions of the ventral and dorsal walls, containing peptic cells whose granules are still PAS-positive, the granules in all the peptic cells lying next to the pyloric area of the stomach have lost their PAS-positive component (fig. 5, A). In sections from areas further away from the pylorus, islands of peptic cells containing PAS-positive granules appear situated near the crypts of the tubules (fig. 4, B), whilst the peptic cells in the bases and bodies of the glands contain only PAS-negative granules. In sections still further from the pylorus only the peptic cells in the bases of the glands (fig. 4, B) contain PAS-negative granules, until finally, on the greater curvature of the stomach (and the adjacent walls) at a site opposite the oesophageal opening, only small islands of peptic cells containing PAS-negative granules occur and these are deep in the bases of the glands (fig. 4, B). These cells are amongst other peptic cells whose granules are PAS-positive (as already reported, Menzies, 1958).

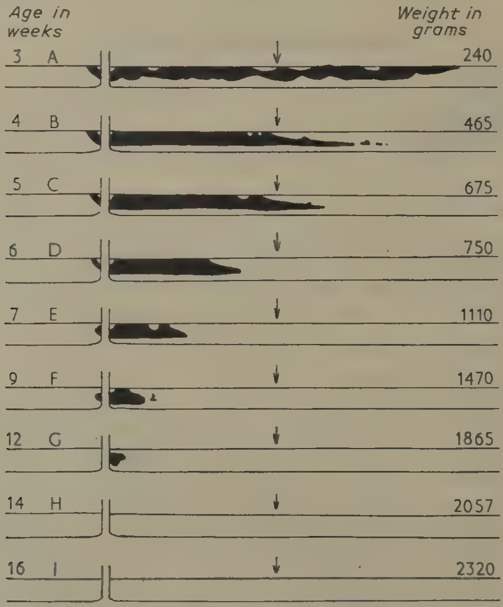

FIG. 4. Diagram of a series of young rabbits from 3 to 16 weeks old, showing (black) the distribution of peptic cells containing granules which are PAS-positive. The diagram represents the oesophageal opening with the lesser curvature to the left and the greater curvature to the right, each end being the pylorus. The arrow is at the site on the greater curvature opposite the oesophageal opening. (Compare fig. 1.)

The 'area' of peptic cells whose granules have lost their PAS-positive component is widest on the greater curvature of the stomach and becomes progressively narrower as it proceeds up the ventral and dorsal walls of the stomach, as is shown in fig. 5, A.

It might be appropriate to stress here that fig. 4 represents peptic cells ontaining granules in a PAS-positive phase. All peptic cell granules from . weeks up to and including the adult stage react positively with Bowie's nethod and show a pattern of distribution not essentially different from that hown in fig. 3.

Animals from 5 to 12 weeks old. There is a progressive loss of the PAS omponent of the peptic granules starting in the cells in the bases of the lands and proceeding up the tubules (fig. 4, C–G), and starting in the cells ear the pylorus and on the greater curvature and proceeding slowly across the ventral and dorsal walls of the stomach to the lesser curvature and more quickly along the greater curvature towards the oesophagus (fig. 5, B–E). The first peptic cells on the lesser curvature to lose their PAS-positive granules are seen at the sixth week (fig. 5, B). Finally, at the eleventh or twelfth week only a few peptic cells remain whose granules are PAS-positive. These cells are situated on the greater curvature near the oesophagus and in some animals they are seen (fig. 5, E) on the ventral and dorsal walls of the stomach around the oesophagus.

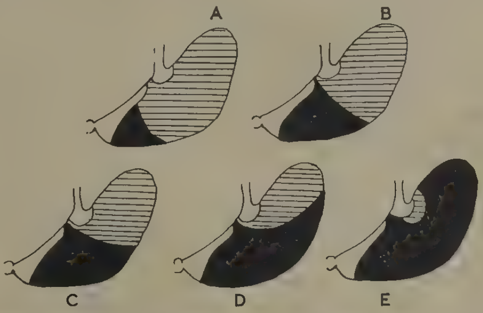

FIG. 5, A–E. Diagrams from animals aged (A) 4, (B) 6, (C) 7, (D) 9, (E) 11–12 weeks. Black represents peptic cells containing PAS-negative granules. Parallel lines represent peptic cells containing PAS-positive granules. Note that in both sets of peptic cells the granules are Bowie-positive.

The changes described above are correlated more closely with the weight of the animal than with its age: animals of the same weight, whatever their age, show similar changes. Furthermore, although the loss of the PAS-positive component of the peptic granules is progressive as already described, it is an irregular progression. There are some sections (from all the animals described above) that show no PAS-positive material in the peptic cells, whereas other sections from the same *blocks* treated at the same time show a few 'islands' of peptic cells whose granules are still PAS positive.

Animals from 14 to 16 weeks old, and those fed on pyronin. Apart from a very few granules in an occasional peptic cell, no PAS-positive material is seen in peptic cells in any of the sections examined from animals over 12 weeks old (or weighing more than 1,900 g—see fig. 4, H–I).

With animals of the same weight, the results observed on those that had been fed on pyronin are similar to those of the normal animals.

DISCUSSION

As already stated, it has been reported from the examination of sections of the gastric mucosa which had been taken from the greater curvature of the stomach opposite the oesophageal entrance, that the loss of the mucinogen phase of the peptic granules was completed by the end of the sixth week after birth. The present investigation confirms that statement in so far as that particular area of the stomach is concerned, but shows that in other areas the change is not completed until the end of the twelfth week. The importance must therefore be stressed of examining sections from all areas of an organ before concluding that serial changes take place within its cells at any specific time. But if this is impracticable, as is usually the case, it would seem essential that all sections to be compared should be taken from similar anatomical sites on the organ concerned.

It is fortunate that the rabbit was chosen for the present series of studies, for in this animal the loss of the mucinogen component of the peptic granules occurs slowly and at a relatively late and well-developed stage of the gastric mucosa, and can therefore be easily observed. It does, however, occur in other mammals, as, for example, in man and the rhesus monkey (Menzies, 1958), and in the rat, mouse, hamster, cat, and dog (personal observations). But with some it is difficult to obtain the material necessary to carry out a complete investigation; with others (rat and mouse) it occurs very early in development, before the gastric tubule has attained any great length, and insufficient cells are present to show any order of loss; whilst with yet others the histological picture is complicated either by a deep-seated layer (mouse) or an irregular distribution (cat and dog) of mucous neck cells, which themselves contain PAS-positive mucinogen granules at all stages. It is not immediately obvious why in the rabbit the peptic granules should lose their mucinogen component in the order in which they do. It is unlikely to be related to the onset of peptic activity, for pepsin has been demonstrated in the gastric juice of the rabbit at 1 to 2 weeks old (Hammarsten, 1874), and furthermore in some mammals, for example the guinea pig and porcupine, most, if not all, of the pepsinogen granules retain their mucinogen component even at the adult stage.

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The Ligament of the Lucinacea (Eulamellibranchia)

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SUMMARY

The basic structure of the opisthodetic ligament of the Lucinacea consists primarily f inner layer, anterior and posterior outer layers, and periostracum. This is secondarily xtended by fused periostracum and fusion layer. Fused periostracum extends as far s the posterior adductor muscle and, except in the Ungulinidae, to the anterior dductor. The fusion layer covers the posterior outer layer and a little beyond. Only n *Loripes lucinalis* is the fusion layer to be found anteriorly. Below the umbos the nner layer, posterior outer layer, and overlying fusion layer are split. This split can e explained in terms of the growth and form of the shell. The anterior outer layer ills the split and extends to the anterior limit of the lunule. The ligament, external in he Ungulinidae, becomes progressively more internal. At the same time the posterior imit of the outer layer becomes modified as a result of the elongation of the outer urface of the outer mantle fold between the pallial lobes in the depth of the posterior nantle embayment. The Lucinidae are most specialized in this respect where, in *L. ucinalis*, a tongue of tissue divides much of the ligament horizontally. The Thyasiridae, he remaining family of the group, occupies an intermediate stage in this specialization.

INTRODUCTION

RECENT studies by Owen, Trueman, and Yonge (1953), Trueman (1953, 1954), Owen (1953, 1958, 1959), and Yonge (1953, 1957) on a wide range f species of Bivalvia make clear the basic form of the adult ligament and the nantle secreting it. Primarily the ligament is composed of an inner layer overed by anterior and posterior outer layers and the periostracum (fig. 7, . 35). The inner layer is secreted by the epithelium of the mantle isthmus, he outer layers are secreted by the outer surface of the outer mantle fold within the depths of the mantle embayments at either end of the mantle sthmus, and the periostracum is secreted by the inner surface of the outer mantle fold. This primary ligament may be secondarily extended anteriorly and/or posteriorly by fusion of the mantle margins and may involve (1) extension by periostracum, where the inner surfaces of the outer mantle folds fuse to form a single periostracal secreting surface, or (2) extension by fusion layer, where the outer surfaces of the outer mantle lobes are united.

Yonge (1957) suggests that 'each natural group of lamellibranchs has a characteristic pattern of mantle fusion involving the ventral margins, the siphons and the ligament'. In a recent investigation into the basic form of the Lucinacea the ligament was examined to see whether its structure was comparable to that of other ligaments described by the above workers and whether it showed characteristics peculiar to the group. In addition to providing answers to these queries this study indicates a pattern of evolution of the ligament that is similar to and probably associated with other evolutionary

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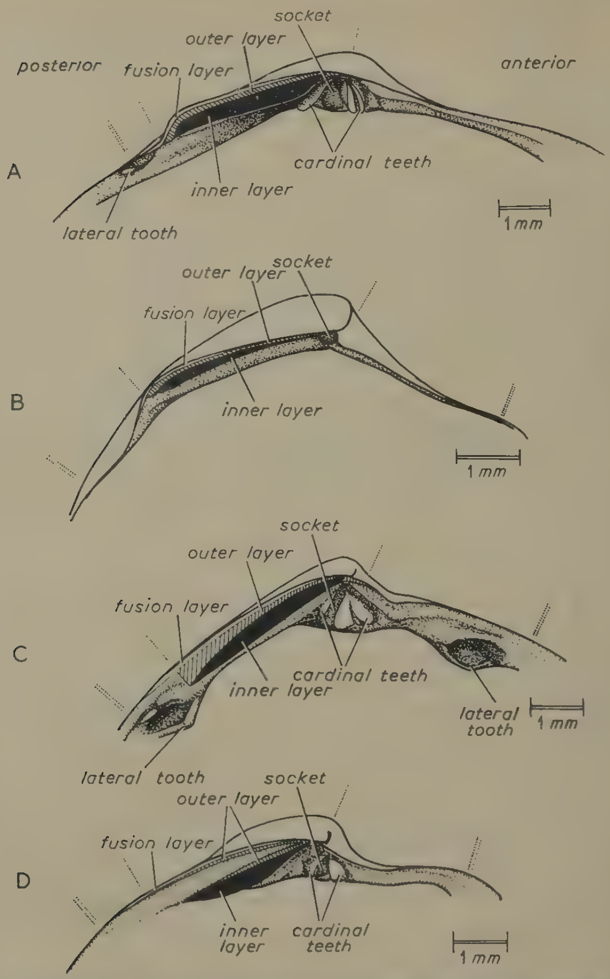

FIG. 1. Lateral views of the hinge structure of the left valves of A, *Diplodonta punctata*; B, *Thyasira flexuosa*; C, *Codakia costata*; and D, *Loripes lucinalis*. Dotted single lines mark the limits of the primary ligament and dotted double lines mark the limits of the secondary extensions of the ligament. The differentiation of the outer layer into anterior and posterio parts is shown in figs. 2 to 5.

rends in this group (Allen, 1958). It also provides information concerning he evolution of the ligament of lamellibranchs generally. The nomenclature roposed by Owen, Trueman, and Yonge (1953) has been followed.

THE HINGE STRUCTURE OF THE LUCINACEA

Three families, the Ungulinidae, the Thyasiridae, and the Lucinidae, are rouped together and form the Lucinacea. They show an evolutionary equence in the development of characteristic morphological features and abits, the Ungulinidae the least and the Lucinidae the most modified Allen, 1958). Although 13 species were examined (Allen, 1958, p. 421), for larity and conciseness reference will be largely restricted to *Diplodonta unctata* Say (Ungulinidae), *Thyasira flexuosa* (Montagu) (Thyasiridae), and *odakia costata* d'Orbigny and *Loripes lucinalis* Turton (Lucinidae).

The hinge teeth of most species examined are poorly developed. The ngulinidae and Lucinidae have two cardinal teeth below the umbo of ach valve. The larger (anterior in the left valve, posterior in the right) is edge-shaped and may be medially grooved (fig. 1). The larger tooth of the ight valve fits behind that of the left. The second smaller cardinal tooth is educed to a slight ridge and forms one wall of the socket into which the arger tooth of the opposite valve fits; i.e. the posterior wall on the left valve nd the anterior wall on the right valve. A very poorly developed posterior ateral tooth may be present in the Ungulinidae. Both anterior and posterior ateral teeth may be present in the Lucinidae. The anterior tooth is usually etter developed than the posterior (*Phacoides*, *Lucina*, *Divaricella*). Lateral eeth are most prominent in species of *Codakia* and absent in *Lucina* (*Lori-inus*) *chrysostoma* and *Loripes lucinalis*. *Thyasira flexuosa* (Thyasiridae) bears very small tooth below the umbo of the right valve. The left valve is othless.

The ligament, in contrast with the hinge teeth, is well developed in the ucinacea. The primary ligament extends from the anterior end of the lunule o a point approximately two-thirds of the way to the posterior adductor uscle. It is an opisthodetic ligament. The secondary ligament may extend s far as the anterior and posterior adductor muscles (figs. 1–5). The basic orm and structure of the ligament closely resemble that of *Tellina tenuis* Trueman 1949) and of *Glauconome rugosa* recently described by Owen 1959). Owen (1959) points out that the structure of the ligament is dependent n the degree of pallial fusion while the form is dependent on the growth attern of the shell. Each shell increment is laid down as an open coaxial gure (gnomon). The ligament of the Lucinacea, being opisthodetic, has a ingle point of minimal growth near the anterior end.

Ungulinidae. The structure of the ligament of *D. punctata* and of this genus enerally is shown in fig. 2. Posteriorly, between the limit of the outer layer nd the anterior margin of the posterior adductor, there is a short extension f the primary ligament by fusion layer and fused periostracum. The ligament s external and, in transverse section, the periostracum, fusion layer, and

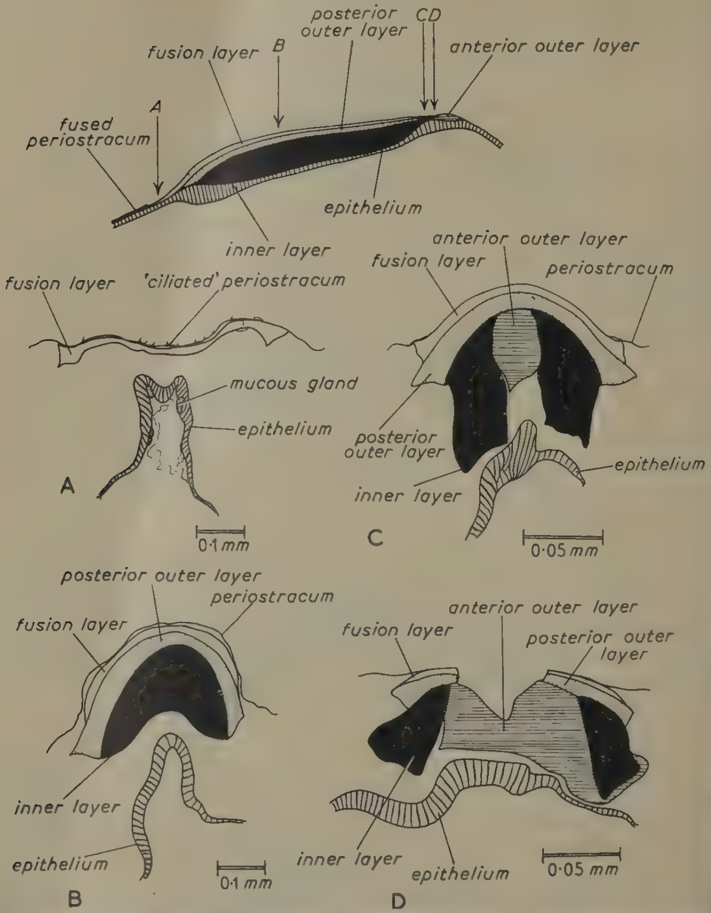

FIG. 2. Semi-diagrammatic representation of transverse sections through the ligament and dorsal margin of the mantle of *Diplodonta punctata*. The levels from which the sections were taken are indicated by arrows over a sagittal section of the same ligament viewed from the right side.

posterior outer layer form an inverted U, the centre of the U being filled with inner layer. Anteriorly below the umbos the ligament is split and bifurcated in the sagittal plane. The extent of the split is slight, approximately 200μ. Sections show that posteriorly only the inner layer is split, while further forward the posterior outer layer, fusion layer, and periostracum are divided (fig. 2, C, D). The anterior outer layer (previously termed the cardinal ligament by Trueman, 1949) fills the split and extends beyond to the anterior limit of

e lunule. In section the anterior outer layer and the fusion layer are so
milar in appearance as to cast some doubt as to the identity of this layer (see
34). However, the anterior outer layer is secreted by the outer surface of
e outer fold of the mantle within the depths of the anterior mantle embay-
ent and sections show that there are no mucous glands present in this
creting epithelium. Mucous glands are present under the fusion layer and
eriostracum. There is no secondary anterior extension to the ligament in
e Ungulinidae.

The secretory epithelia of the different layers of the ligament indicated in
gs. 2 to 5 are similar to those described by Yonge (1953) for *Pinna carnea.*
he cells secreting the outer layer are taller than those secreting the inner
yer. The former are particularly well developed posteriorly at the point of
aximum growth. The cells secreting the inner layer are cuboid, thus differ-
g somewhat from those in *Pinna*. No mucous glands are present in the
ithelia underlying the primary ligament. The cells secreting the fusion
yer, although columnar, are not as tall as those secreting the outer layers,
d their cytoplasm is not as granular as that of the cells below the primary
gament. Mucous glands are present in the epithelium-secreting fusion layer
d fused periostracum. As Yonge (1953) observed in *Pinna*, there is no sharp
emarcation between the various epithelia.

Thyasiridae. The ligament of *Thyasira flexuosa* differs little from that
escribed above for the Ungulinidae. In cross-section the posterior outer
yer and fusion layer no longer form an inverted U (fig. 3) and little is seen
f the ligament externally. Posteriorly the fusion layer extends somewhat
rther than in the Ungulinidae and is, relatively, a much thinner layer. The
osterior outer layer is thicker than that in *D. punctata* and forms a sharp
osterior boundary instead of merging gradually to the fusion layer. The
rupt termination is reflected in the secreting epithelium, where the tall
ranular cells form a compact group with an indentation that coincides with
e junction of inner and outer layers (fig. 3). The anterior outer layer differs
ttle from that in the Ungulinidae, but there is secondary extension by fused
eriostracum as far as the anterior adductor. The anterior bifurcation of the
rimary ligament is more pronounced than that in the Ungulinidae, but, as
the latter family, the split is bridged by the anterior outer layer (fig. 3, F).
he sagittal split is continued posteriorly as a cavity between the inner and
osterior outer layer (fig. 3, D). This cavity is lozenge-shaped in cross-section
nd lined with anterior outer layer secreted by a tongue of epithelium. In *T.*
exuosa, at the tips of the bifurcation, the inner layer is lateral to the posterior
uter layer and not ventral (compare figs. 3, D, F, and 2, D).

A similar but very much greater bifurcation occurs in *Glossus* (Owen,
953). In this case Owen relates the progressive anterior splitting to a large
ngential component of growth. There is no anterior secondary extension of
e ligament in *Glossus*. There is little doubt that the bifurcation of the liga-
ent in the Lucinacea is a result of the tangential component of growth.
hile this component is very large in *Glossus* it is small in the Lucinacea and

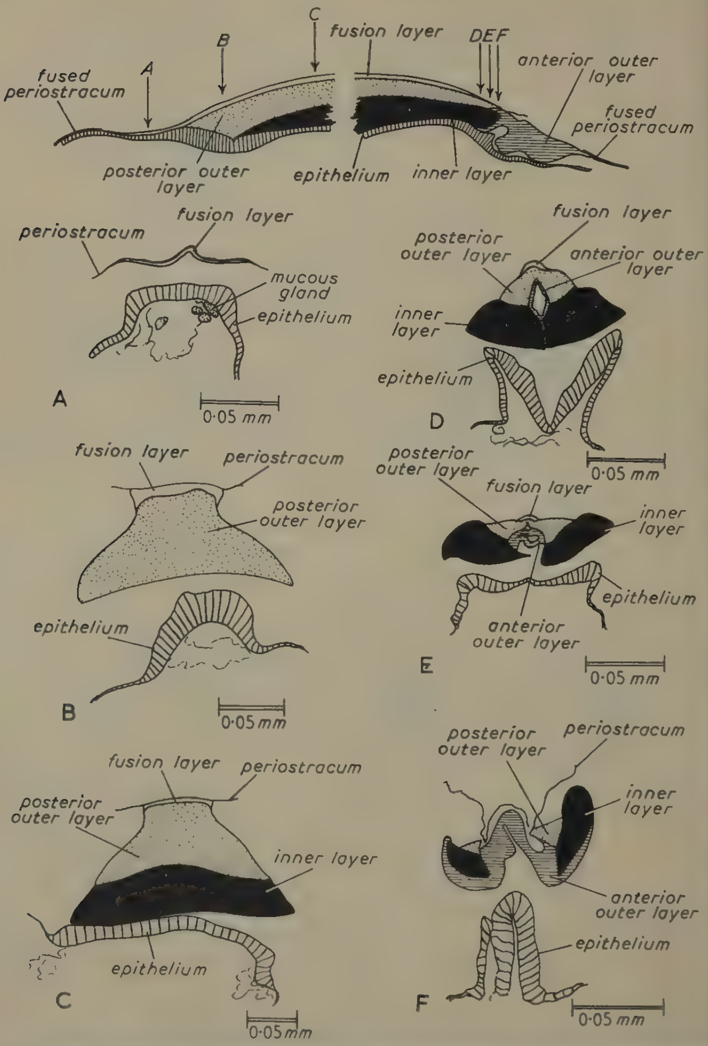

FIG. 3. Semi-diagrammatic representation of transverse sections through the ligament and dorsal margin of the mantle of *Thyasira flexuosa*. The levels from which the sections were taken are indicated by arrows over a sagittal section of the same ligament viewed from the right side.

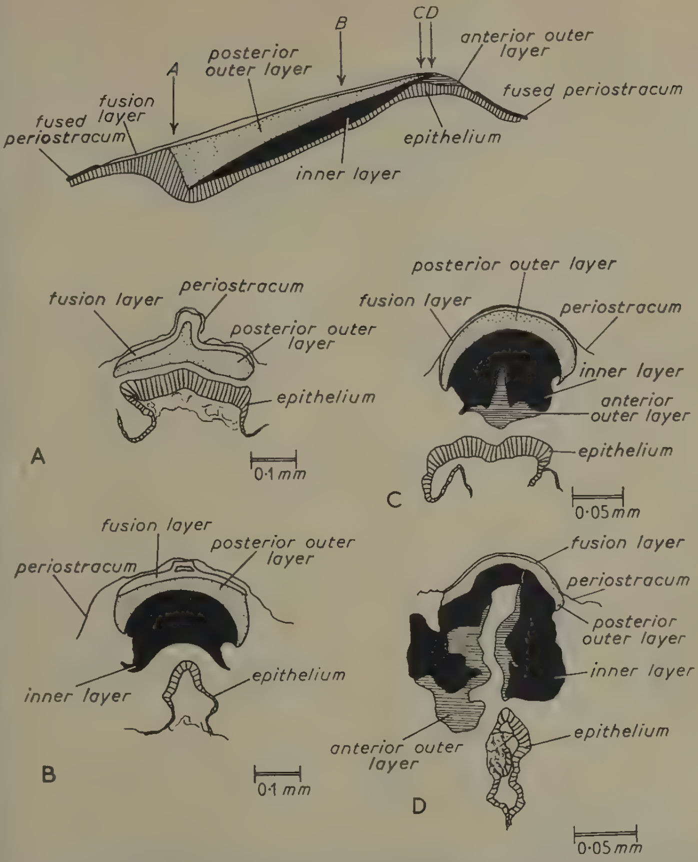

FIG. 4. Semi-diagrammatic representation of transverse sections through the ligament and dorsal margin of the mantle of *Codakia costata*. The levels from which the sections were taken are indicated by arrows over a sagittal section of the same ligament viewed from the right side.

the resulting split is considerably shorter. Unlike *Glossus* the outer lobes of the mantle anterior to the primary ligament are fused with the consequent extension by periostracum and, in the case of *L. lucinalis* (p. 34), by fusion layer. Unlike *Glossus* (Owen, 1953, fig. 3, E–H), the split does not involve the anterior outer layer. Sections, and observations on the intact ligament of the Lucinacea, suggest that the anterior outer layer is laid down in a more fluid form than the other layers. It appears to function as a cement or filling repairing the split ligament. Anterior outer layer is also present ventral to the inner layer and occasionally dorsal to the posterior outer layer, forming a small convoluted 'knob' at the anterior end of the primary ligament. Trueman (1949) describes a similar condition for *Tellina tenuis*. There is no sign of successive layers of conchyolin as is seen in the other layers of the ligament.

The greater part of the region of bifurcation in fully grown animals corresponds to the entire ligament of the larvae and early post-larvae. The split also coincides with the region of minimum growth in the older shells. As successive shell increments are laid down, the two regions representing the valves of the early post-larva are carried farther apart beyond the limit of normal maximum gape (fig. 6). This, as Trueman (1950) postulates for *Mytilus edulis*, may in itself be responsible for the split and reinforces the effect of the tangential component of growth. The divergence of the two halves of the split will be due to the effect of the tangential component alone. The lateral position of the inner layer at the tips of the bifurcation as seen in *Thyasira flexuosa* is a result of a large constant angle of growth (Thompson, 1942).

Lucinidae. All species of the Lucinidae examined, except *L. lucinalis*, have a ligament that is similar to that described for the Thyasiridae. The characteristic, sharply defined posterior limit of the outer layer is even more pronounced, ending abruptly at right angles to the fusion layer. Similarly, the corresponding indentation of the epithelial layer is much more pronounced than in *T. flexuosa* (fig. 4). The extent of the anterior split is similar to that described for *Diplodonta*. The split initially affects the internal surface of the inner layer (fig. 4, c). The anterior outer layer is secreted between the split and extends to the limit of the lunule. Secondary extension of the ligament is similar to that of *Thyasira*. Anteriorly fused periostracum extends as far as the anterior adductor muscle. Posteriorly the fusion layer extends almost to the posterior adductor muscle. The short distance between the limit of the fusion layer and the muscle is roofed by fused periostracum.

In *L. lucinalis* (fig. 5) modification of the posterior limit of the primary ligament is extreme. A tongue of epithelium reaches anteriorly almost as far as the umbo and divides much of the ligament horizontally into a long, narrow, external portion and a shorter, broad, scoop-shaped internal portion. This epithelium secretes posterior outer layer dorsally and ventrally, and shell laterally. The external part of the ligament is composed of fused periostracum, fusion layer, and a comparatively thick posterior outer layer, while the internal part is composed of inner layer and a thin outer layer, probably comparable to layer 1*c* described by Trueman (1949) for *Tellina tenuis*. The outer layers of each part are joined together at the umbos (fig. 5). The form of this ligament is correlated with the elongation and folding of the outer surface of the outer mantle fold between the pallial lobes in the depth of the posterior mantle embayment. This is not an isolated example, for elongation of the mantle epitheleum occurs in the Semelidae (Trueman, 1953). In the Semelidae there is a similar horizontal division of an opisthodetic ligament but here inner layer is present at each side of the external part.

In *L. lucinalis* the growth pattern of the inner layer is clearly seen. Each lamina or growth increment is thick posteriorly but thin ventrally (fig. 5), so

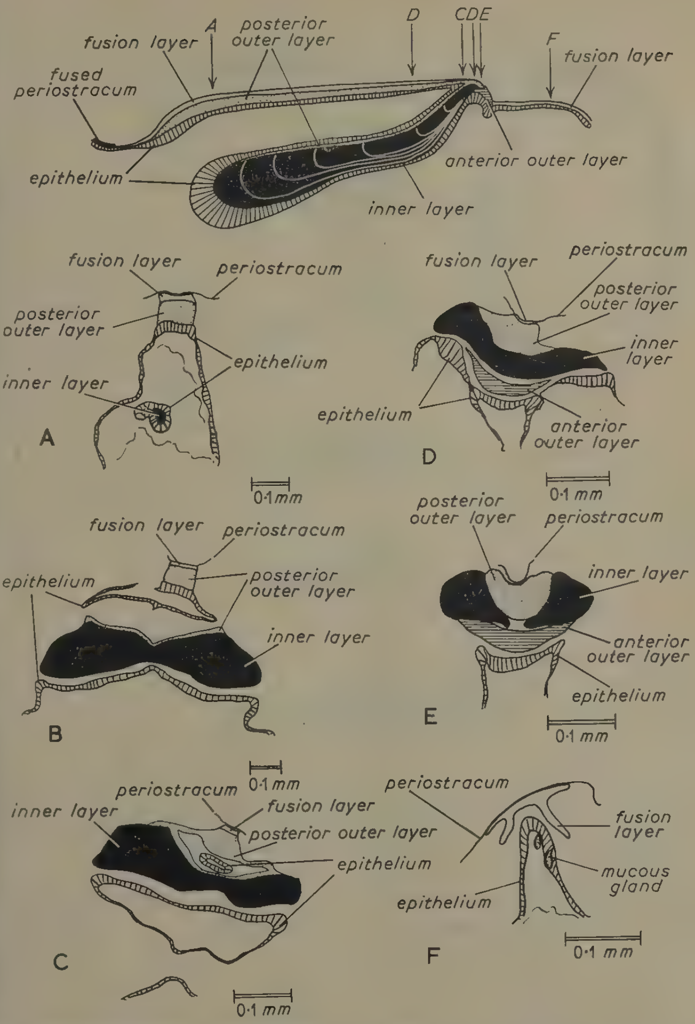

FIG. 5. Semi-diagrammatic representation of transverse sections through the ligament and dorsal margin of the mantle of *Loripes lucinalis*. The levels from which the sections were taken are indicated by arrows over a sagittal section of the same ligament viewed from the right side.

that in transverse sections other than at the extreme posterior part it appears that there are two zones, the upper with vertical and the lower with horizontal layering. In trichrome staining the lower zone stains blue while the upper is pale pink or colourless.

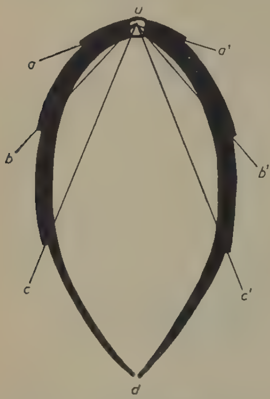

FIG. 6. Transverse section through the shell of a four-year-old bivalve. *ua*, *ab*, *bc*, *cd* represent annual increments of growth, while the angles *aua'*, *bub'*, *cuc'* represent the present gape of what were the valves of one-, two- and three-year-old shells.

The ligament is split anteriorly in much the same way as other members of the Lucinidae. Anterior outer layer cements the split and extends to the anterior limit of the lunule. The ligament is secondarily extended as far as the anterior adductor muscle. Fusion layer and fused periostracum are both involved. There is no fused periostracum anterior to the fusion layer. The fusion layer and anterior outer layer are so similar in staining and refractive properties that it is difficult to distinguish the junction between them. In *L. lucinalis* the fusion layer forms two long parallel ridges adjoining the valves of the shell, the ridges being connected by a thin dorsal covering (fig. 5, F). Posterior secondary extension of the external portion of the ligament is similar to that in other Lucinidae.

DISCUSSION

Of the Lucinacea, the ligament of the Ungulinidae is the least modified and may be said to represent the basic form of the group. There is no secondary extension anterior, and little posterior, to the limits of the primary ligament. Modification in the Thyasiridae and Lucinidae includes secondary extension by fused periostracum as far as the anterior and posterior adductor muscles. Fusion layer extends beyond the posterior limit of the posterior outer layer but never as far as the posterior adductor muscle. Only in *L. lucinalis* is fusion layer present anterior to the ligament. Further modification includes the elongation of the outer surface of the outer mantle fold in the depth of the posterior embayment. At its maximum, in *L. lucinalis*, a tongue of tissue divides the ligament horizontally into internal and external portions. An evolutionary sequence can be postulated (fig. 7).

The anterior end of the ligament is split at the point of minimum growth and involves inner layer, posterior outer layer, and fusion layer. The extent of the split varies with the tangential component of growth and the constant angle of the valves. The split is filled with anterior outer layer, which acts both as cement and as a protection to the thinnest part of the ligament. Observations suggest that this layer is secreted in a more fluid form than other layers. The anterior outer layer probably has protective function in all opisthodetic ligaments at the point of minimum growth.

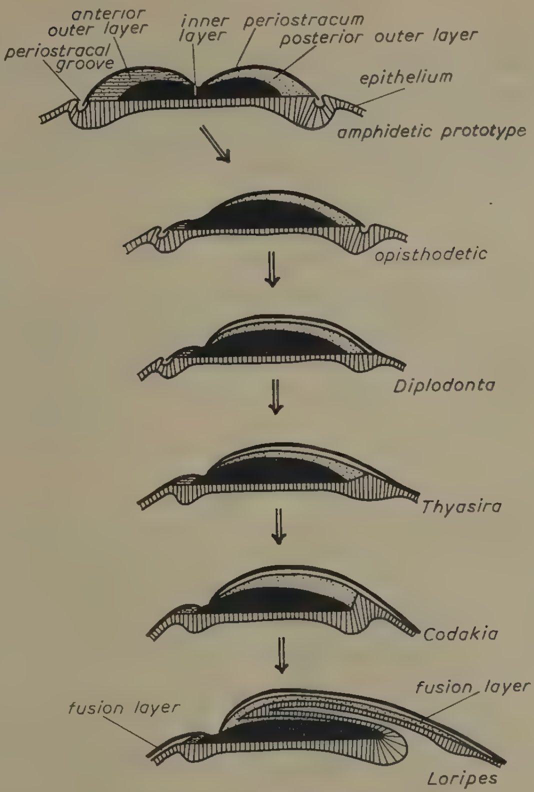

FIG. 7. The possible course of evolution of the ligament of the Lucinacea. Diagrammatic sagittal sections, seen from the left side. The various layers of the ligament correspond to those labelled in figs. 2 to 5.

I wish to thank Professor C. M. Yonge, C.B.E., F.R.S., for his advice and encouragement and Dr. G. Owen for his criticism of the manuscript. I am also indebted to the John Murray Committee of the Royal Society for a grant that made this work possible.

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An Occurrence of Hermaphroditism in *Rana catesbiana*

By W. KEITH O'STEEN

(*From the Department of Anatomy, University of Texas (Medical Branch), Galveston, Texas, U.S.A.*)

With one plate (fig. 1)

SUMMARY

Hermaphroditism is rather uncommon in the bullfrog, *Rana catesbiana*. A case is described here. Among the features of the unusual urogenital system were male secondary sexual characters (large tympanic membrane, vocal sacs, nuptial pads), testicular tissue with developing and mature male gametes, and accessory ducts (vasa efferentia, seminal vesicles). Müllerian ducts and large ovarian masses containing oöcytes and mature ova were also present. Oöcytes were observed in the lumina of seminiferous tubules.

INTRODUCTION

MANY descriptions of true hermaphroditism have been reported for several genera and species of Amphibia. Hermaphroditism in frogs has been described and summarized in the bibliographies of Crew (1921), Witschi (1921), Christensen (1929), and Bayliff (1940). From the literature it was concluded that intersexuality in amphibians, at least in some species, was fairly common. However, this type of sexual abnormality appears to be rather unusual in the bullfrog, *Rana catesbiana* Shaw. Bayliff (1940) cited only 5 previously reported cases of intersexuality in this species (Clemens, 1921; Chidester, 1926; Behney, 1933; Moore and Byers, 1938).

The specimen reported in this paper was found during routine examination of approximately 300 adult male and female bullfrogs.

GROSS STRUCTURE

The specimen was 147 mm long (snout to anus). It was originally classified as a male from its secondary sexual characteristics. The nuptial pads were well developed, the vocal sac openings were large, and the diameter of the tympanic membrane corresponded to that of other males of comparable size.

On examination of the internal organs the animal appeared to be a normal female, but on further examination the male sexual apparatus also was found in normal size and shape, with the following exceptions. The structure of the testes, which measured approximately 12 mm in length and 5 mm in diameter, was interrupted in the anterior half by a large mass of ovarian tissue extending into its inner portion. Vasa efferentia and modified mesonephric ducts (seminal vesicles) apparently were normal.

The ovaries, with lengths of approximately 80 mm and widths of 30 mm, contained hundreds of mature ova, showing distinctly marked animal and vegetal poles. The oviducts were normally developed as compared with

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females of comparable size. All reproductive structures were bilaterally symmetrical.

HISTOLOGICAL STRUCTURE

The ovotestes were removed from the coelom and, after most of the ovarian tissue had been removed, were fixed, embedded, sectioned longitudinally at 10 μ, and stained with haematoxylin and eosin. Sections made through mature ova, which were poorly fixed, were unsatisfactory owing to flaking of the yolk material.

The testicular portions of the gonads were normal in appearance in certain regions (fig. 1, A, C). Seminiferous tubules contained spermatocytes in different phases of meiosis, and Sertoli cells in close relationship to mature spermatozoa. However, in other testicular regions the seminiferous tubules contained oöcytes of various sizes in addition to spermatozoa (fig. 1, B, C). The connective tissue of this portion was apparently normal.

The ovary contained mature ova and all stages of developing oöcytes. Degeneration of many of these cells was observed. Thick connective tissue, not usually found in the normal gonad, was present in the areas where the capsule of the large ovarian masses was continuous with the capsule of the testicular portion.

Although ova were observed within the seminiferous tubules there was no indication of male structures within the ovary.

DISCUSSION

The hermaphrodite bullfrogs described here and by the others already mentioned were similar in some but not in all of their abnormal structures. They differed primarily in the degree of intersexuality.

The specimen described here apparently had the capacity to fertilize itself, since spermatozoa and ova were observed within the ovotestes; however, no proof can be offered here that this actually had occurred.

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FIG. 1 (plate). Photomicrographs from longitudinal sections of ovotestis. Formalin, haematoxylin, and eosin.

A, ovotestis with oöcytes scattered through the testicular portion. Ova show flaking of the yolk cytoplasm owing to poor fixation.

B, seminiferous tubule, cross-section, with cells in meiosis, mature spermatozoa, and oöcytes within the lumen.

C, section showing seminiferous tubules, some with and some without oöcytes in the lumina.

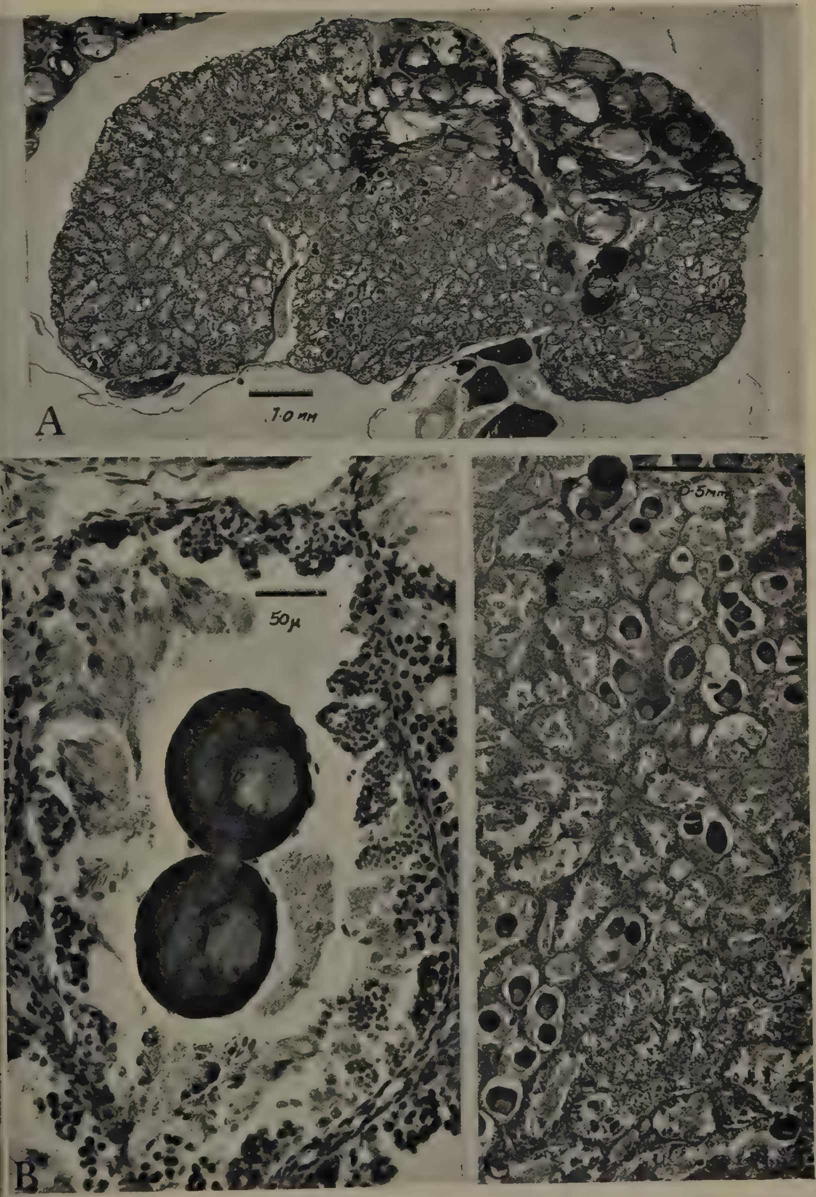

FIG. 1
W. K. O'STEEN

A Modification of the 'Hermann PO' Method for Mitochondria

By BARBARA M. JORDAN-LUKE

(From the Cytological Laboratory, Department of Zoology, University Museum, Oxford)

With one plate (fig. 1)

SUMMARY

Tissues are fixed in Hermann's fluid, postosmicated at 34° C, and embedded in n-butyl methacrylate. Sections are cut with an oblique knife on a sliding microtome, attached to glass slides with albumen, and mounted in DPX or Canada balsam.

Mitochondria are shown in black against a colourless, transparent cytoplasm. They retain their original form. Preparations made in this way are superior to those made by the original Hermann PO technique in the remarkable clarity of the mitochondria and the absence of distortion in the tissue as a whole.

It is confirmed that the presence of acetic acid at 5% in the fixative in no way damages the mitochondria.

HERMANN'S fluid is an excellent fixative for mitochondria, provided that the tissue is postosmicated; and if the postosmication is carried out for several days at 34° C, the mitochondria are blackened (Baker, 1957). This is probably the easiest and most satisfactory way of making mitochondrial preparations for light microscopy. Filamentous mitochondria retain their form.

In the original technique, paraffin sections were used. It occurred to me to try embedding instead in methacrylate. The blackening of the mitochondria was not affected, and the smaller degree of general shrinkage of the tissue resulted in even better preparations.

Dr. J. R. Baker and I have devised new methods of cutting methacrylate sections for light microscopy, and of attaching them to glass slides. These methods have only been briefly mentioned in print (Baker, 1960). It is thought that a fuller account of them might be of use to others and this is presented here as a part of the new technique.

The following fluids are required:

1. Hermann's fluid (1889). This is most easily made up thus:

Distilled water	0·8 ml
Chloroplatinic acid, 5% aq	0·3 ml
Osmium tetroxide, 2% aq	0·4 ml
Acetic acid, 20% aq	0·5 ml

2. Osmium tetroxide, 2% aq, for postosmication.
3. *n*-butyl methacrylate for embedding, prepared as for electron-microscopy.
4. Adhesive albumen (Baker and Jordan, 1953), for attaching sections to slides.
5. Ethyl acetate, for the solution of methacrylate polymer.

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The technique is carried out as follows:

I. Fix pieces of tissue not more than 3 mm thick in Hermann's fluid for 24 h.

II. Wash in running water (or repeated changes) for 24 h. Give a final wash ($\frac{1}{4}$ h) in distilled water.

III. Postosmicate for 3, 4, or 5 days at 34° C. The best period must be discovered by experiment. (Postosmication at 37° C gives much less satisfactory preparations.)

IV. Wash for several hours in running water (or repeated changes).

V. Embed in methacrylate, by the same process as is used in electron microscopy. Since larger pieces are used than in electron microscopy, the period in the monomer should be extended (up to 24 h, with several changes).

VI. Attach the block to a sliding microtome by means of a metal chuck especially made to hold it. (If the polymer is particularly hard, the block may be soaked overnight in 70% ethanol.)

VII. Cut sections at 3 to $5\,\mu$ in the same way as collodion sections are cut, that is, with an oblique plano-concave knife (concave surface upwards); but the knife should be tilted at a more oblique angle than for cutting collodion sections. Flood the knife with 70% ethanol. The sections may be stored in 70% ethanol.

VIII. Transfer the sections to distilled water.

IX. Attach the sections to glass slides exactly as though they were paraffin sections, by the use of adhesive albumen (Baker and Jordan, 1953).

X. When the sections are dry, put the slides in ethyl acetate, and leave them until the methacrylate has dissolved.

XI. Bring the slides through absolute ethanol to xylene, and mount in DPX or Canada balsam.

No claim is made that the method is specific for mitochondria. For instance, the chromophil part of the acroblast is blackened (fig. 1, D, *ac*). In most tissues, however, the mitochondria are the chief objects that are blackened. The zymogen granules of the pancreas, which are deeply coloured by most mitochondrial methods, are untouched, and indeed are almost invisible (fig. 1, A). The form of the mitochondria is remarkably well preserved, as the photomicrographs show (fig. 1). They are quite black against a colourless, transparent cytoplasm. It has not been found possible to obtain a distinctive

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FIG. 1 (plate). All four photomicrographs represent tissues treated by the Hermann PO methacrylate technique.

A, pancreas of mouse (exocrine cells).

B, intestinal epithelium of mouse.

C, testis of mouse. The labelled cell is a primary spermatocyte.

D, testis of cricket (*Acheta domesticus*).

ac, acroblast; *ap*, apical group of mitochondria; *bas*, basal group of mitochondria; *fb*, fre border of intestinal epithelium; *lum*, lumen of intestine; *mit*, mitochondrion, mitochondria *Neb*, mitochondrial Nebenkern; *nuc*, nucleus.

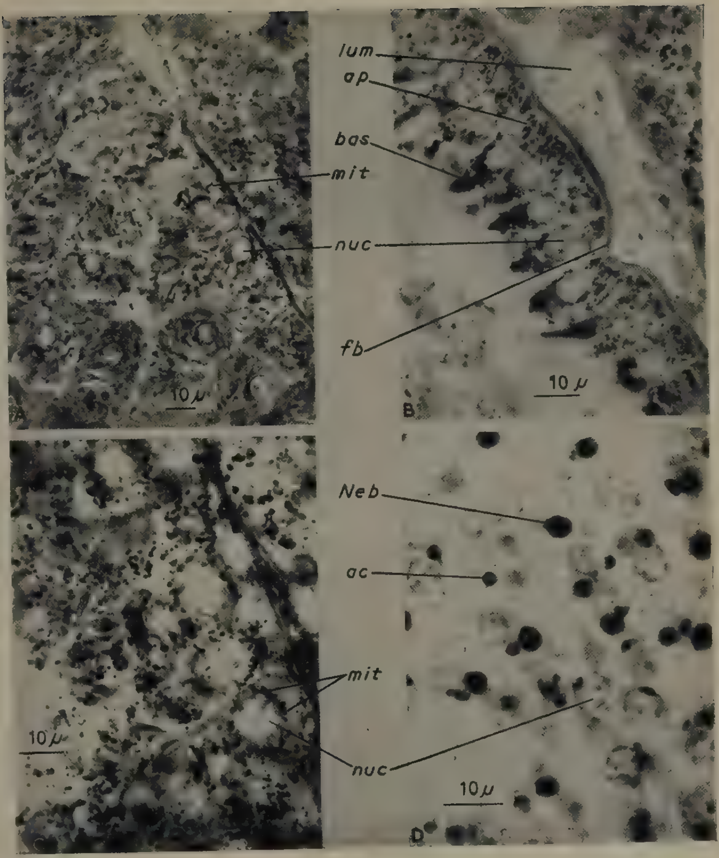

FIG. 1

B. M. JORDAN-LUKE

yeing of chromatin, and it is best to leave the sections unstained. The nuclei an be distinguished, though neither the nuclear membrane nor the chromatin blackened. The best part of the preparation is often a little below the surace, and for this reason very minute pieces should not be used as a general ule. The intestinal epithelium of mammals, however, shows mitochondria ell, even if directly exposed to the fixative. The general fixation of the tissue good, and there is little distortion by uneven shrinkage.

There are a few tissues (notably mammalian liver) that are rendered brittle y the Hermann PO technique, and do not give good methacrylate sections.

The success of this technique proves conclusively once again that mitohondria are not damaged (far less destroyed) by the inclusion of acetic acid t 5% in a fixative, provided that the tissue is postosmicated before being ubjected to the action of lipid solvents.

I am grateful to Dr. J. R. Baker, F.R.S., for his kind help in writing this aper.

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The Use of Fixed and Stained Sections in Quantitative Studies of Peripheral Nerve

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SUMMARY

A technique which has been widely used in quantitative studies of peripheral nerve volves fixation in Flemming's fluid, embedding in paraffin wax, and staining with ematoxylin. An appraisal of the size changes induced by a standardized form of this chnique has been made. Observations have been confined to changes in the thickness d external diameter of the internodal compact myelin sheath of the non-fasciculated rvus gastrocnemius medialis of the adult rabbit.

For a comparison of external diameters, sections from adjacent parts of the chosen rve were prepared (*a*) by the standardized Flemming-Wolter technique; (*b*) by the pid freezing technique, previously shown to reproduce accurately the dimensions esent in fresh teased unfixed nerve-fibres. There was no significant difference in stribution of external diameters between sections prepared in the two ways.

For a comparison of sheath thicknesses, a regression line of 2 × myelin sheath thickss on external diameter was derived from each type of preparation. As external ameter was not appreciably affected by processing, a direct comparison of these gression lines could be made. It was found that regardless of external diameter or iginal sheath thickness, processing caused a reduction of sheath thickness of some % as a result of increase in internal diameter. This latter increase ranged from out 50% to under 20% with increasing fibre size.

Single frozen sections were passed through the series of reagents used in the emming–Wolter technique and photographed after each of seven stages. The incipal changes in sheath thickness occurred in the first and last stages of processing. xternal diameter was not appreciably affected whereas the internal margin of the eath was extremely labile. Observations suggest that slight inconsistencies in techque may be accompanied by relatively gross errors when measurements are underken. All stages need careful standardization, the most important in this respect being xation, differentiation, final dehydration, and photographic exposure.

INTRODUCTION

MOST quantitative studies of peripheral nerve have been based on sections which have been fixed and stained. Such methods of preparation e generally assumed to be accompanied by some degree of shrinkage and disrtion (Baker, 1958), which will vary with the particular tissue component nd reagents used.

It has been shown that the proportion of compact myelin to axoplasm in eripheral nerve-fibres varies appreciably not only with external fibre diameter Taylor, 1942; Sanders, 1948; Evans and Vizoso, 1951), but also with the pe of nerve studied (Wendell-Smith and Williams, 1958). It is possible at the size changes of axoplasm and myelin induced by a variety of reagents e comparable and proportionate so that the relation between them is unfected. However, in view of the marked difference in the ultrastructure of

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these two components, this possibility seems remote, and on *a priori* grounds differential effects may be postulated.

A technique which has been widely used involves fixation in Flemming's fluid, embedding in paraffin wax, and staining with haematoxylin. Much important comparative work has been carried out by a standardized form of this technique. The error involved has proved unimportant in this context. Moreover, most workers have limited their studies to external fibre diameter, and as will be shown this dimension is not appreciably affected by the technique. However, modern theories of conduction (Rushton, 1951) concern themselves in part with the ratio between the diameter of the axon and the thickness of the ensheathing structures, so that for their verification a knowledge of the effect of the standardized technique on these dimensions is necessary. Thus attention is focused not only on possible changes in the external diameter of the compact myelin sheath but more particularly on changes in internal diameter. Further, the validity of assuming that estimates of internal diameter performed on fixed and stained preparations provide a reliable index of axon diameter (e.g. Gasser and Grundfest, 1939) must be considered. In addition the importance of internal diameter measurements is stressed in relation to the observed differences between nerve-fibres of muscle and skin of the same external diameter, and to possible variations in the ratio of thickness of the myelin sheath to diameter of the axon along the length of the fibre (Evans and Vizoso, 1951; Sunderland and Roche, 1958).

The literature dealing with the size changes during processing has been reviewed by Sunderland and Roche (1958). A number of different techniques have been assessed by as many methods, but the majority of workers have confined their studies to changes in external diameter. Sanders (1948) studied the changes in both internal and external diameter as a result of a modified Weigert technique (Gutmann and Sanders, 1943). The principal stages of this technique are similar to those considered here. However, the nerve studied was the peroneal nerve of the rabbit, in which there are nerve-fibres of both muscle and skin. His basis of reference was the freshly teased nerve-fibre, and he made no allowance for the magnifying effect of the myelin lens system (Williams and Wendell-Smith, 1958).

MATERIAL AND METHOD

The nervus gastrocnemius medialis (N.G.M.) of the rabbit was used throughout the present investigation. Adult rabbits were used; no account was taken of breed, sex, or weight. The use of lipid-soluble inhalation anaesthetics was avoided and intravenous nembutal was used. The appropriate part of the sciatic nerve and its branches was removed in one piece. Sections from a short segment including the N.G.M. at about 3 cm proximal to its muscle were prepared in two ways.

(i) 5- or 6-μ sections of the fresh nerve were prepared by a rapid freezing technique (Williams, 1959). The sections were irrigated with a standard volume of normal saline and photographed immediately without a cover-slip, polarized light. The volume of saline, 2 drops, has been found by experi-ent to be the most satisfactory. A larger volume often introduces an un-esirable degree of meniscus curvature and is more sensitive to extraneous air rrents, which produce streaming and interference with the polarization otics.

In such sections the dimensions of the compact myelin sheath do not differ gnificantly from those present in fresh teased fibres (Wendell-Smith and illiams, 1959).

(ii) The second method of preparation is given in full because it is believed at slight variations in technique may prove important when measurements e contemplated (Causey, 1948; Evans and Vizoso, 1951).

1. Remove the nerve carefully with the epineurium intact, and attach under slight tension to a frame prepared from a library card.
2. Fix for 24 h in 19 ml of freshly prepared Flemming's fluid, with reduced acetic acid, in a 20 ml tightly stoppered bottle.

   Fixative solution used

   1% chromic acid in distilled water—15 ml.
   2% osmium tetroxide in distilled water—4 ml.
   glacial acetic acid—1 drop (25 mgm).

3. Dehydrate without washing for 1 h each in 70%, 90%, and 95% alcohol, and absolute alcohol (2 changes).
4. Clear in cedarwood oil, 15 h.
5. Vacuum-embed in paraffin wax (m.p. 56° C), 2 h.
6. Section at 5 or 6μ and attach to slide.
7. 3% potassium dichromate at 37° C, 6 h.
8. Rinse, stain in 100 ml Wolter's haematoxylin for 15 h at 37° C.

   Wolter's haematoxylin

   haematoxylin 1 g—dissolve in 10 to 20 ml of absolute alcohol.
   distilled water to 100 ml.
   glacial acetic acid 2 ml.
   Filter before use.

9. 3% potassium dichromate, 5 min at room temperature.
10. Differentiate in 0·25% potassium permanganate, 5 sec.
11. Flood with Pal's bleach, 5 min.
12. Wash, dehydrate (95% and absolute alcohol, 1 min in each), clear in xylene (2 changes, 1 min in each), mount in Canada balsam.

Photography. All photographs were taken with a Leitz Panphot apparatus, reliminary focusing being on the ground glass screen and final critical ocusing by means of the telescope.

As in previous experiments the fresh sections were photographed by light om a carbon arc source, and the exposure time was standardized at 3 sec. he fixed sections were photographed with light from a tungsten filament mp source, with an Ilford micro 3 filter No. 404 and an exposure time of

10 sec. Ilford R. 20 quarter-plates were developed in I.D. 11 fine grain developer for 6 min at room temperature, and subsequently enlargements ×1,000 and ×2,000 diameters were made on Kodak W.S.G. 2. S. paper and developed in D. 163 for 2 min. After processing, the prints were allowed to dry between sheets of Fotonic paper. A squeegee was not used. A micrometer scale was photographed and enlarged at each session.

The optimum exposure times were determined by a comparison between the dimensions of the direct visual image and the dimensions of the photographic images produced after different exposure times. Ten fibres between 11 and 21 μ in external diameter were selected, and measurements of internal and external diameter made directly on the slide with a screw ocular micrometer to the nearest 0·125 μ, and indirectly with a ruler to the nearest 0·25 μ on photographic enlargements after exposures of $3\frac{1}{2}$, 5, 7, 10, 15, and 20 sec. The ratio of internal to external diameter was used as a basis for comparisons by the χ^2 test.

Comparison of external diameters. The non-fasciculated part of the N.G.M. was removed as above, and carefully cut into two segments. Sections from the two adjoining cut surfaces were prepared by the freezing technique and the Flemming–Wolter technique respectively. Estimates of external diameter were made by two methods. A Perspex disk inscribed with concentric circles 2 mm apart was used to allocate the magnified images of fibres to size-categories differing in diameter by 2 μ steps. This method was chosen initially because it has frequently been adopted by other workers in this field. Since minor differences might not be apparent when a 2 μ grouping was used, a more accurate estimation was made for one N.G.M. by taking the mean of two measurements at right angles made with a 0·5 mm scale. Histograms were constructed and a comparison of the distributions made by the χ^2 test.

Comparison of sheath thicknesses. Sections were prepared by the two techniques and estimates of external and internal diameter were made on photographs at final magnifications of 1,000 diameters (10 to 20 μ fibres) and 2,000 diameters ($<$ 10 μ fibres). For each fibre the estimator was the mean of two measurements made at right angles. The maximum error of the measuring technique was ±4%.

For each type of preparation a scatter diagram was constructed by plotting external diameter against 2 × sheath thickness. Regression lines and standard errors of estimate were calculated.

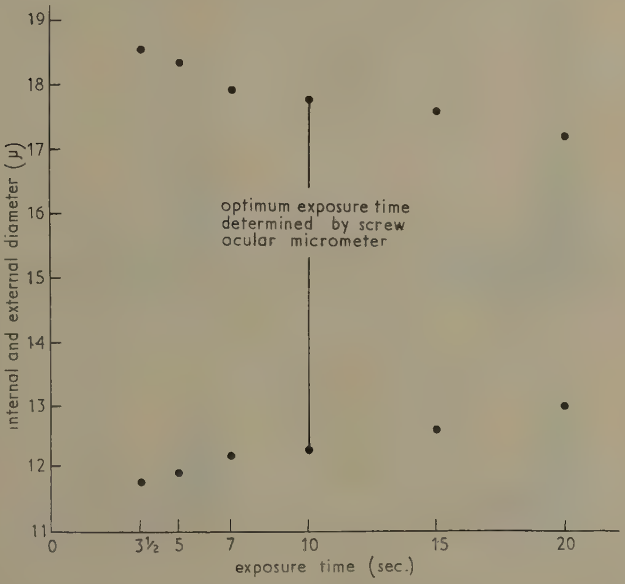
The results from frozen sections and fixed stained sections were compared and the changes in sheath thickness and internal diameter demonstrated graphically.

Illustration of lability of sheath dimensions during processing. Frozen sections were prepared and photographed after each of the following successive stages:

1. Physiological saline.
2. Flemming's fluid (15 min).
3. 70%, 90%, 95%, and absolute alcohol (15 min each).

4. Cedarwood oil (15 min).
5. Molten paraffin wax (15 min) and return to xylene (15 min).
6. Through the alcohols to water.
7. Wolter's haematoxylin staining; dehydration, clearing, and mounting in Canada balsam.

Twenty fibres, all approximately 15 μ in external diameter, were followed



IG. 1. Internal diameter and external diameter plotted against exposure time. Each point epresents the mean measurement for the same 10 fibres after the specified exposure time had been used in the production of a negative.

hrough the successive stages by measurements of internal and external iameter. The mean changes in dimensions were used to construct a diagram.

RESULTS

Exposure time. It was found that the thickness of the myelin sheath de-reased with increasing exposure time; that this decrease was regular and due o changes in both internal and external diameter (fig. 1). The ratios of internal o external diameter for the 10 fibres as determined with the screw ocular icrometer were taken as the best estimates. The ratios as determined on hotographic images after 10 sec exposure did not differ significantly from hose estimates. This and other results are given in table 1.

Comparison of external diameters. It was found that there was no significant difference in distribution between sections prepared by the two techniques (table 2).

TABLE 1

Significance tests for difference between direct measurements and those on photographs

Exposure in seconds	χ^2	*Degrees of freedom*	*Probability*
3·5	60·21	9	$<0·001$
5·0	37·31	9	$<0·001$
7·0	21·36	9	$<0·02$
10·0	11·67	9	$>0·20$
15·0	31·47	9	$<0·001$
20·0	80·75	9	$<0·001$

TABLE 2

Significance tests for difference between fresh and fixed-stained fibre distributions

Nerve	*Grouping* (μ)	χ^2	*Degrees of freedom*	*Probability*
N_1	2	14·70	9	0·10
N_2	2	9·39	9	0·40
N_3	2	11·41	9	0·25
N_1, N_2, and N_3	2	35·50	29	0·20
N_1	1	21·61	18	0·25
N_1	0·5	40·62	35	0·25

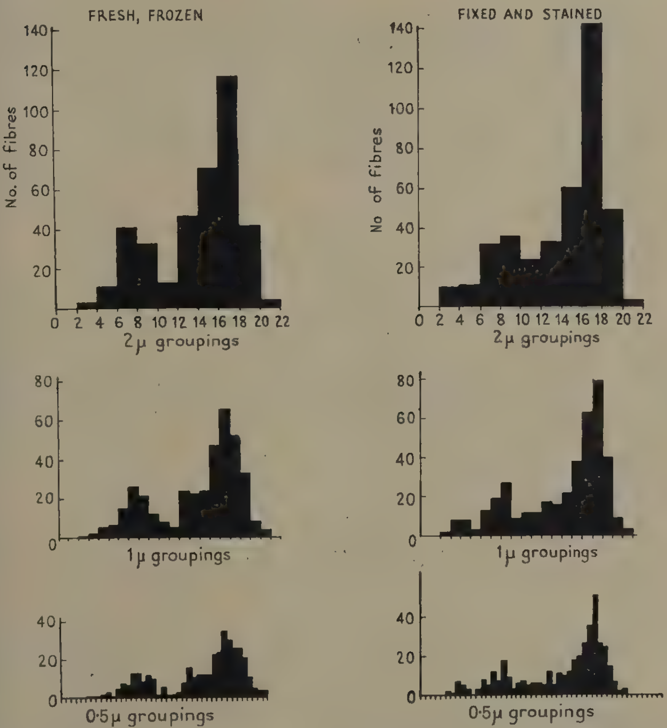
Histograms for the N.G.M. which was examined in more detail are shown in fig. 2.

Comparison of sheath thicknesses. It having been demonstrated that external diameter was not appreciably different after processing, a direct comparison of the regression lines of sheath thickness on external diameter gave a measure of reduction in sheath thickness.

Fig. 3, A is a scatter diagram of 2 × sheath thickness against external diameter for 204 fibres from fresh frozen sections. A calculated regression line and relevant statistics are given in fig. 3, B. A similar scatter diagram and regression line for 172 fibres from the fixed and stained preparation are given in fig. 4, A, B.

The regression lines for fresh frozen sections and for fixed stained sections are compared in fig. 5. Comparison of these lines shows that processing resulted in a reduction of sheath thickness of some 20% regardless of external diameter (fig. 6, A) or original sheath thickness (fig. 6, B). This reduction in sheath thickness was the result of an increase in internal diameter. It will be noted that within the range of fibres studied, as fibre diameter increased, the increase in internal diameter changed from some 50% to under 20% (fig. 6, C).

Lability of sheath dimensions. The results of this investigation are illustrated fig. 7. The principal changes in sheath thickness occurred in the first and t stages of processing. External diameter was not appreciably affected, ereas the internal margin of the sheath was extremely labile. A typical -μ fibre as seen in a section prepared by the standard Flemming–Wolter chnique is included for comparison.



G. 2. Histograms of an N.G.M. of the rabbit, prepared by the two methods from adjacent rtions of the nerve, presented in the usual 2-μ groupings and also in 1-μ and 0·5-μ group- ngs. The essential similarity of the distributions persists throughout the three groupings.

DISCUSSION

It is axiomatic that for quantitative histology a standardized technique is ccessary. Different aspects of the technique requiring standardization will considered.

The selection of a particular nerve and site is important in an investigation this type. As has been pointed out, a mixed population of fibres to muscle

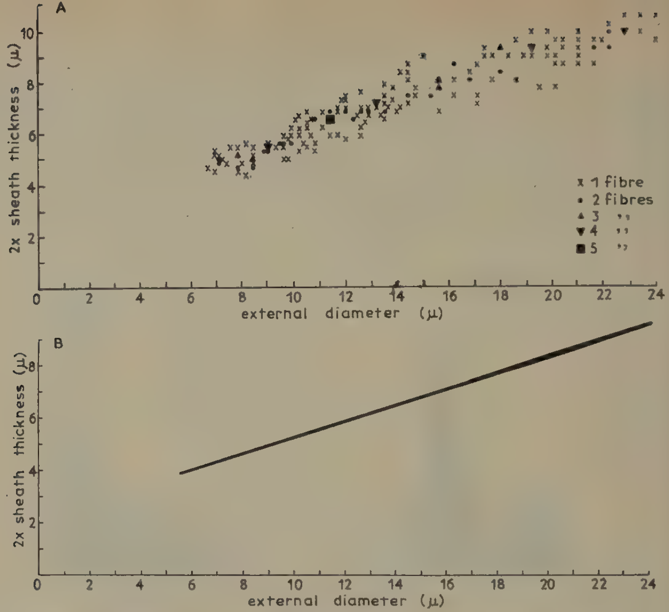


FIG. 3. A, scatter diagram of 2× myelin sheath thickness on external diameter for 204 fibre from frozen sections of the N.G.M. of the rabbit. B, calculated regression line for data fro fig. 3, A. Correlation coefficient, 0·9291; regression coefficient, 0·3099; standard error of esti- mate of sheath thickness from external diameter, 0·5287μ.

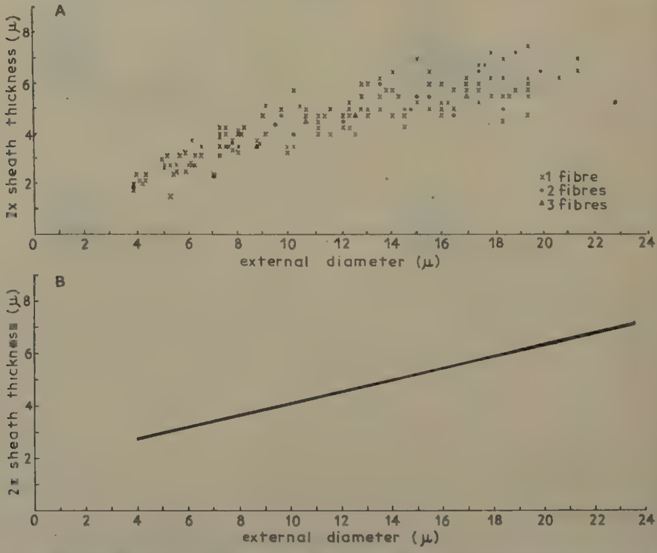
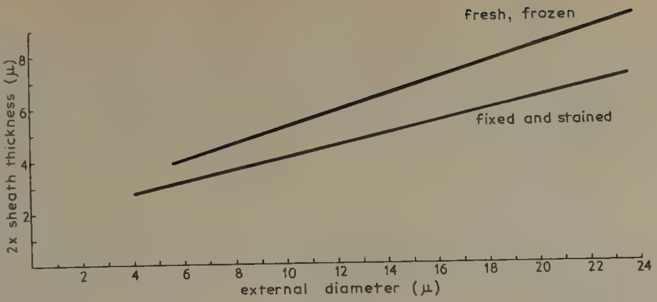
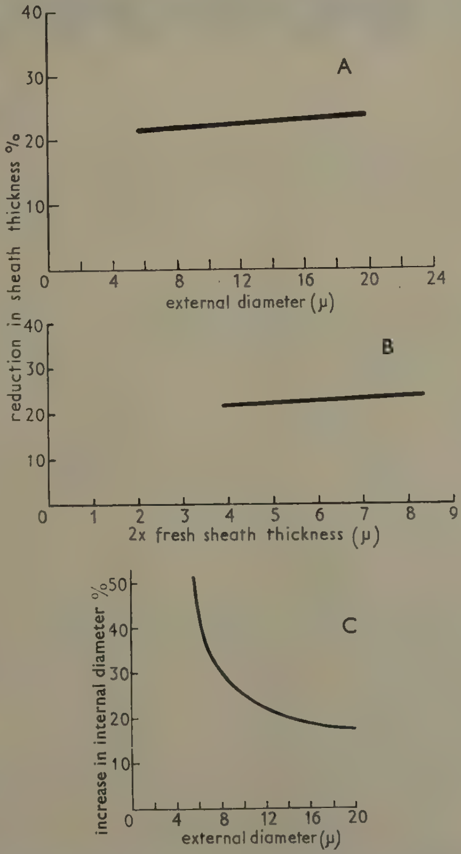


FIG. 4. A, scatter diagram of 2× myelin sheath thickness on external diameter for 172 fibre from Flemming–Wolter preparations of the N.G.M. of the rabbit. B, calculated regressio line for data from fig. 4, A. Correlation coefficient, 0·9500; regression coefficient, 0·2292 standard error of estimate of sheath thickness from external diameter, 0·4154μ.



G. 5. Calculated regression lines for data from figs. 3, A and 4, A, with statistics as in figs. 3, B and 4, B.



IG. 6. Curves derived from fig. 5. A, curve relating reduction in 2 × sheath thickness due to rocessing, to external fibre diameter. B, curve relating reduction in 2 × sheath thickness due › processing, to fresh sheath thickness. C, shrinkage factor. A curve relating percentage increase in internal diameter due to processing, to external diameter.

and skin should not be used. Fasciculation has been shown to be accompanied by branching of myelinated fibres (Quilliam, 1956), which is clearly undesirable. The evidence concerning peripheral tapering of myelinated nerve-fibres has been reviewed by Sunderland (1958). Causey (1948) and Evans and Vizoso (1951) found no evidence of tapering along the greater part of the N.G.M. The latter authors, however, present data suggesting a gradual increase in the proportion of myelin peripherally. For these reasons, our sections were taken from a short segment (<5 mm) of the non-fasciculated portion of the N.G.M. of adult rabbits, 3 cm proximal to its point of entry into the muscle.

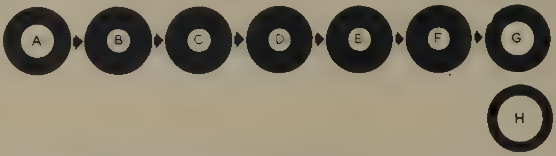


FIG. 7. A series of scale diagrams illustrating the lability of the myelin sheath during successive stages of processing. A, fresh, frozen section in normal saline. B, after fixation in Flemming's fluid. C, after dehydration. D, after clearing. E, after immersion in molten paraffin wax and return to xylene. F, after rehydration. G, after haematoxylin staining and final dehydration, clearing, and mounting. H, for comparison, a typical fibre of similar external dimensions prepared by the standardized Flemming–Wolter technique.

When removing the nerve for processing we have found it important not to dissect fine nerves from the parent trunk nor to remove the epineurium, as these manœuvres lead to stretching and distortion. Removal of the appropriate part of the sciatic nerve and its branches in one piece avoids this.

When attaching the nerves to card frames, attempts were made to restore their original length (Fernand and Young, 1951).

The effects on penetration, autolysis, and fibre contour of variations in the composition of Flemming's fixative have been studied by Causey (1948), who found that 1 drop of glacial acetic acid delivered from a standard pipette gave optimal results. We have used a 25-mgm drop in 19 ml of chrome-osmium mixture for each specimen.

Evans and Vizoso (1951) found that the most critical factor in the staining process is the time allowed for differentiation in the potassium permanganate bath. This in turn is dependent upon density of staining. Staining in 100 ml of Wolter's haematoxylin at 37° C for 15 h allows the differentiation time to be fixed at 5 sec. Wolter's haematoxylin is made immediately before use and the variability of natural ripening processes is eliminated.

The experiment demonstrating the lability of sheath dimensions during processing showed that the major changes occurred during initial fixation and final dehydration and clearing before mounting. Frequently the latter stages are the least well controlled with respect to time. During this experiment reagents were poured directly on to the freshly cut surface of a microscopic section, which was attached to a slide. This situation is not directly comparable with the standard Flemming–Wolter technique and thus has little quantitative value. Nevertheless, it is believed to provide information of a qualitative type concerning the site of maximum lability (the internal border of the compact myelin) and the stages of processing principally concerned.

General measures have been taken to standardize the photographic technique according to the work of Rexed (1944). However, Evans and Vizoso (1951) point out that a serious error may be introduced by variations in the intensity of the light and in the exposure time. Using direct projection on to bromide paper they found marked differences in the ratio of myelin area to axon area when exposures were made for 15 sec and 30 sec, and therefore standardized their exposure time, presumably at an arbitrary level. We have determined and used the exposure time which leads to an image of dimensions comparable to those determined with a sensitive screw ocular micrometer. It is interesting to note that this exposure time corresponded to that chosen empirically and quite independently by an experienced photographer to give a 'normal' negative after development over the recommended interval for the emulsion used. No experiments with different emulsions were made.

Some importance attaches to the finding that external diameter was little affected by the reagents used in the Flemming–Wolter technique in view of the wide use of similar techniques for quantitative studies, involving external diameter only.

Studies involving internal diameter have been more limited. They include aspects of growth (Evans and Vizoso, 1951) and regeneration (Sanders, 1948), as well as theoretical (Rushton, 1951) and practical (Gasser and Grundfest, 1939) functional studies. In this connexion it is necessary to consider the reduction in sheath thickness of some 20%, associated with increases in internal diameter varying with fibre size between 20 and 50%. Clearly a measurement of the internal diameter of the myelin sheath made on a fixed-stained preparation bears no simple relation to the axon diameter.

Measurements made on such preparations do, however, give useful information on the behaviour of compact myelin under the conditions of the experiment. It has been shown that the Flemming–Wolter technique causes a constant size-change per unit of myelin thickness (fig. 6, B). This may be regarded as an expression of systematic change affecting the lamellar subunits of myelin. Such a view receives strong support from the work of Finean (1958), who used X-ray diffraction techniques to study quantitatively the effects of reagents on the ultrastructural distances of nerve myelin. His basis of reference was the diffraction pattern of the freshly dissected nerve. The percentage change per radial repeating unit (after dehydration) was of a similar order to that described above. Furthermore, a number of substantial changes of proportion were shown to be reversible. This accords well with the observations on sheath lability during processing illustrated here.

We wish to thank Professor R. Warwick for his interest in this work, Mr. A. N. Finch for technical assistance and photography, and the Department of Medical Illustration, Guy's Hospital Medical School, for preparing figs. 2–7. This investigation has been supported by grants from the Central Research Fund, University of London.

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Patterns of Myo-neural Junctions and Cholinesterase Activity in the Muscles of Tadpoles of *Xenopus laevis*

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(*From the Department of Anatomy, Cambridge*)

With two plates (figs. 1 and 9)

SUMMARY

A simultaneous coupling azo dye technique has been used to reveal the distribution of cholinesterase activity in the musculature of the developing tadpole of *Xenopus laevis*. The use of inhibitors and a less convenient but more specific histochemical technique confirmed that only true cholinesterase distribution was being demonstrated; and a study of silver-impregnated material proved that this azo dye technique provides a very convenient method of following the development of the patterns of myo-neural junctions in the striated muscles of this tadpole. A wide variety of patterns is seen in the various muscles: in the axial musculature the muscle-fibres become innervated at their ends from myocommatal plexuses and never acquire endings along their length; broad muscular sheets, as in the walls of the branchial and abdominal cavities, are also first innervated terminally from the septa but later acquire secondary patterns of innervation over their surfaces; while in long narrow muscles the main innervation is along the lengths of the fibres. These different patterns of innervation are correlated with the functions of the various types of muscle. It is suggested that terminal innervation may be a special adaptation to permit rapid establishment of neurogenic activity, the pattern of endings of the more usual type forming when the need for precisely co-ordinated reflexogenic activity arises. In some muscles, the azo dye technique reveals a profuse multiple innervation of the fibres which are assumed to be of the so-called 'slow type' known to exist in some amphibian muscles.

INTRODUCTION

THE developing patterns of myo-neural junctions in the voluntary musculature of the tadpole of *Xenopus laevis* are described in this paper. The survey is based largely on the study of whole larvae or of dissections in which the azo dye coupling technique described by Lewis (1958) has been performed. Such preparations reveal the distribution of esterases throughout the tissues, which within the developing muscles may be assumed to be cholinesterases. The results have now been correlated with the examination of section series of tadpoles impregnated with silver by Bodian's method. Comparison of the results obtained by these two means has shown that the pattern of esterase-positive sites in the developing muscles is identical with that of the myo-neural junctions.

Preliminary accounts of these observations have already been published, both in the technical paper (Lewis, 1958) and elsewhere (Lewis and Hughes, 1957). In these papers, attention was drawn to the fact that the motor innervation of the larval myotomes is confined to the ends of the fibres. These

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observations are now confirmed, and this type of innervation is compared with that of other larval muscles.

Material and Methods

The methods used to obtain and culture the *Xenopus* larvae have been briefly described by Tschumi (1957).

In a preliminary series of experiments most of the histochemical methods said to reveal cholinesterase were tried. The simultaneous coupling azo dye technique with α-naphthyl acetate as substrate appeared to be the most suitable for our purpose and unless otherwise stated all the observations recorded in this paper were obtained with some modification or other of this general technique. It soon became clear that the accepted frozen-section technique was quite unsuitable for a general embryological survey; so a special modification was devised which made it possible to make esterase preparations of thick specimens, even of whole tadpoles. Full experimental details of this modification as applied to *Xenopus* tadpoles have been published elsewhere (Lewis, 1958). A closely spaced series of esterase preparations of whole tadpoles was made covering the period from before hatching right up to metamorphosis. Preparations of individual muscles and of frozen sections were also made to elucidate particular points which required finer definition than could be obtained with this main series. Small pieces of muscle were teased out from some of these esterase preparations and were treated by the Bodian technique so that both nerve-fibres and cholinesterase distribution were shown in the same specimen.

Tadpoles were also fixed and embedded in paraffin, and serial sections silvered by Bodian's protargol method.

Something should perhaps be said about the specificity of the esterase method used. Several of the visceral organs gave intense staining which was clearly due to an ali-esterase rather than a cholinesterase. These tissues were usually removed by dissection before the esterase technique was applied to whole tadpoles. A positive reaction was also given by occasional blood-cells, which were, however, easy to identify under the microscope. With these few exceptions all the sites of activity seen appear to have been due to true cholinesterase. Both eserine and 1:5-bis (4-trimethylammoniumphenyl) pentane-3-one diiodide (a highly specific inhibitor for true cholinesterase (Bayliss and Todrick, 1956)) were used on representative specimens at a concentration which should produce approximately 90% inhibition of true cholinesterase, and the marked reduction of staining produced was quite consistent with such a degree of inhibition. Added to this is the fact that it was usually possible to demonstrate nerve-fibres in association with the deposits of azo dye. Both the indoxyl acetate method of Barrnett and Seligman (1951) and the thioacetic acid method of Crevier and Bélanger (1955) gave results quite consistent with those of the α-naphthyl acetate method but were used only a few times because they appeared to be technically less satisfactory for our particular purpose.

Several modifications of the acetylthiocholine method introduced by Koelle

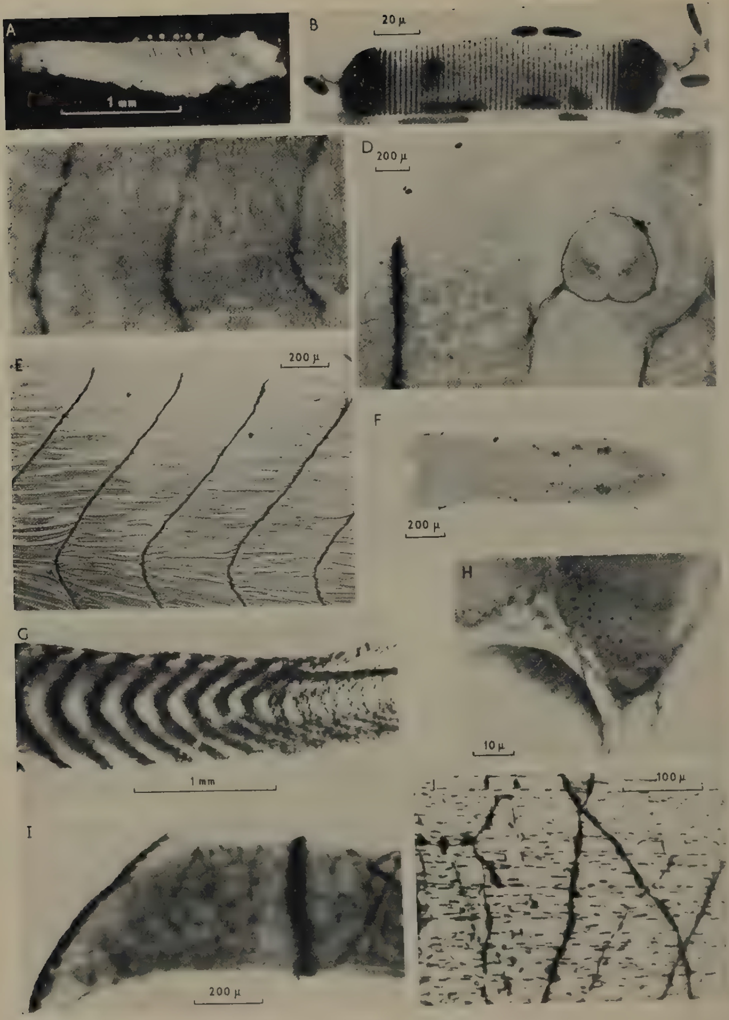

FIG. 1

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and Friedenwald (1949) were tried, but all gave consistently disappointing results when incubation was carried out at 37° C. When the temperature of incubation was reduced to 22° C, however, excellent demonstration of motor nerve-endings was obtained; clearly, the cholinesterase of these tadpoles is much more heat-labile than the corresponding mammalian enzyme. This technique was not used as a routine, however, since it is not so suitable as the azo dye technique for thick specimens. Material from a few tadpoles was treated by the modification described by Shute and Lewis (1959) but with the incubation carried out at room temperature. The results obtained with this modified technique amply confirmed the view that all the sites of azo dye staining discussed in the present paper were sites of true cholinesterase activity.

THE AXIAL MUSCULATURE

Before the embryo of *Xenopus* leaves its jelly coat and becomes a larva, reactions apparently of a reflex type can be evoked. At stage 29 of Nieuwkoop and Faber (1956), some 5 h before hatching in our material, one tadpole responded to pinching of the tail by flexure of only the anterior region of the trunk. After the esterase reaction had been performed on this larva, significant activity was found in the dorsal region of a few of the anterior myocommata (fig. 1, A).

The spinal cord itself first becomes esterase-positive in the preceding stage, N. & F. 28, by which time white matter is already present in the anterior half of the cord (Nieuwkoop and Faber, 1956), but the individual fibres do not yet show any affinity for silver. The youngest larva in which we could recognize simple nerve processes in the myocommata with certainty in a Bodian preparation was one at stage N. & F. 30, and even here the assistance of the

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FIG. 1 (plate). A, a stage 29 tadpole showing the first appearance of esterase activity in the upper parts of 5 of the anterior myocommata, indicated by dots; the other, unstained, myocommatal regions and the intervening rows of muscle nuclei are faintly visible.

B, stage 45; a single myotomal fibre stained by the Bodian technique after the esterase procedure, showing the nerve-fibres and esterase reaction at both ends.

C, stage 35; a 50 μ parasagittal frozen section through trunk myotomes, showing esterase activity confined to the myocommata.

D, stage 50; Koelle preparation, 20 μ transverse section through trunk region, showing the spinal cord with primary motor-cells, a ventral root, and a myocommatal plane on the left.

E, stage 49; Koelle preparation, 20 μ parasagittal section, showing cholinesterase activity confined to myocommatal regions.

F, stage 45; horizontal section through whole brain, showing the motor-cells which innervate the pharyngeal muscles as one intensely stained and two moderately stained groups on each side in the hind-brain.

G, stage 52; tail region showing change in pattern of esterase activity as arrangement of muscle-fibre alters; tip of tail to the right.

H, stage 50; Koelle preparation, 15 μ transverse section through the end of an axial muscle-fibre, showing both a diffuse and a punctate distribution of cholinesterase activity.

I, stage 55; interhyoideus muscle showing primary reaction in median raphe and at lateral edge of muscle, together with the secondary pattern along the lengths of the muscle-fibres.

J, stage 48; constrictor brachialis stained by the Bodian technique after the esterase procedure, showing identity of motor-fibre and esterase distribution of secondary pattern of innervation.

phase microscope was necessary to reveal them. The establishment of reflex behaviour before this stage, however, shows that the rudiments of the peripheral nervous system must be present before we were able to identify them in serial sections.

At stage N. & F. 32, Bodian series show a well-silvered myocommatal plexus in the anterior two-thirds of the trunk (fig. 2), the constituent fibres of which loop around the ends of the muscle-fibres. These nervous elements are the terminals of the spinal ventral roots, the development of which has been described in a previous paper (Hughes, 1959).

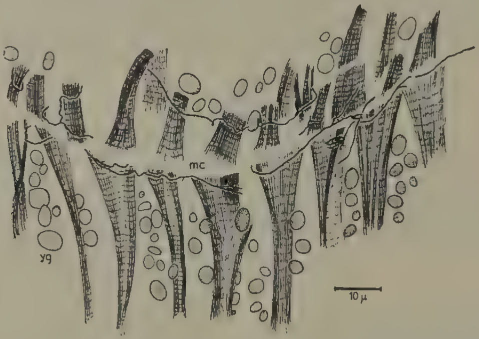

FIG. 2. Sagittal section through axial musculature of trunk of *Xenopus* larva at stage 32, showing plexus of motor-fibres on each side of myocomma. Bodian. *mc*, myocomma; *yg*, yolk granules.

A strong esterase reaction was always observed in the whole of the more anterior myocommata in tadpoles at stages 33–35 (fig. 1, C). At the immediately preceding stages (approximately 30–32) some reaction could always be seen here in all tadpoles which executed swimming movements in response to pinching the tail. The spread of esterase over the anterior myocommata must be a very rapid process since so few specimens were seen in which the process was only partially complete: either activity was confined to the spinal cord or else the most anterior 6 to 10 myocommata showed a reaction over their whole surface.

The myotomes are made up of uninuclear elements which extend between adjacent myocommata. Within each muscle-cell, usually on the mesial surface, is a bundle of myofibrillae which have already become cross-striated in the more mature myotomes. The remainder of the cytoplasm of the cell still contains numerous yolk granules (fig. 1, C).

With the subsequent development of the tail-bud, the larva rapidly grows in length, and the number of myotomes concomitantly increases. In esterase preparations of the whole larva, the number of densely stained myocommata is continuously augmented, but up to stage N. & F. 43, posteriorly there is a gradual fading out of the reaction in the last quarter of the whole series of myotomes. In longitudinal sections at these stages, it can be seen that the youngest myotomes are made up of myoblasts which as yet lack cross-striated myofibrils. Bodian preparations show that an irregular plexus of ventral root-fibres run over the mesial surface of the youngest myotomes, without as yet sending fibres into the myocommata.

Esterase preparations from stage N. & F. 44 onwards show a further development in the pattern of innervation of the axial musculature. A positive reaction now extends through the whole range of myotomes, but in the new and posterior members of the series, the esterase-positive sites take the form of a row of dots on each side of the myocommatal plane, and not of a thin continuous sheet as is seen elsewhere. This more scattered distribution of esterase about the myocommatal planes is due to a different pattern of arrangement of the muscle-fibres. These are now shorter than the inter-myocommatal distance, with long strands of connective tissue attached to each end. The fibres in each myotome are arranged in an overlapping array, each fibre having a cap of esterase and a motor nerve-termination at each end. The change in pattern, which is very abrupt, can be clearly seen in esterase preparations of whole larva which have reached the feeding stage (fig. 1, G). The corresponding Bodian preparations show in the tail region a plexus of motor fibres, extending to 30 or 40 μ on each side of the myocommatal plane (fig. 3). In tracing through the series of motor innervations, one sees an abrupt change from the trunk

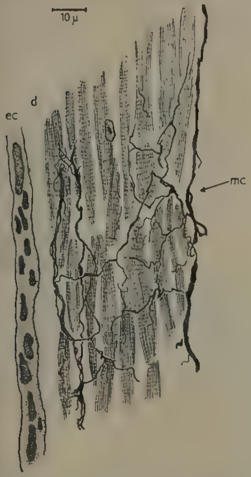

FIG. 3.

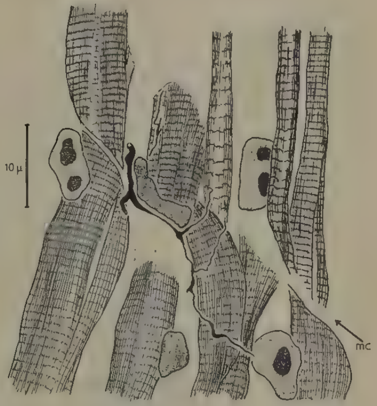

FIG. 4.

FIG. 3. Horizontal section through axial musculature of tail of *Xenopus* larva at stage 45, showing broad plexus of motor-fibres between adjacent myotomes. Bodian. *ec*, ectoderm; *d*, dermis; *mc*, site of myocomma.

FIG. 4. Horizontal section through axial musculature of trunk of *Xenopus* larva at stage 49, showing simple character of nerve-endings at myocommatal boundary. Bodian. *mc*, myocomma.

region where the motor-endings are restricted to a layer of 10 μ in width, to the broad zone of fibres in the tail musculature. This distinction is found throughout larval life; in the trunk the motor-endings remain as simple terminal fibres applied to the bases of the muscle-fibres (fig. 4), while in the tail a branched and plexiform innervation persists. The tip of the tail in the living larva of *Xenopus* continuously exhibits a rapid flickering movement, while the trunk remains immobile for long periods in the undisturbed animal. The spinal ventral roots of the tail region contain a number of giant fibres (Hughes, 1959), the calibre of which is presumably correlated with the conduction of rapid impulses necessitated by the flickering motion of the tail, with which its more elaborate pattern of motor innervation must also be related.

Experiments with the modified Koelle technique confirmed that all the activity seen in esterase preparations of the axial musculature was due to true cholinesterase (fig. 1, D, E). The Koelle technique was in general more limited in application than the azo dye technique, but it did sometimes give much finer detail in sections through particular regions. Thus in transverse sections through the ends of axial muscle-fibres (fig. 1, H) it revealed a punctate distribution of enzyme activity which is highly consistent with the electron-micrographs of this region obtained by Muir (1959). A paper on the fine structure of this type of ending in a number of species, including *Xenopus*, is due to be published shortly by MacKay, Muir, and Peters (personal communication).

The Pharyngeal, Branchial, and Abdominal Musculature

The musculature which can be seen in esterase preparations of the whole larva was studied in a series of specimens from stages N. & F. 40 to 47, and the individual muscles identified as far as was possible from the descriptions given by Edgeworth (1930), Weisz (1945), and Nieuwkoop and Faber (1956). Figs. 5 and 6 illustrate the pattern of esterase-positive sites in the muscles of a 14 mm larva at stage N. & F. 47 in the pharynx, and in the branchial and abdominal cavities. Such preparations were again compared at each stage with Bodian-stained section series.

From stages N. & F. 34 to 39, nerve-branches of the trigemino-facialis group can be traced to the surface of the levator mandibularis, and along the ceratohyal, to each side of which are attached the levator hyoideus and the interhyoideus respectively. All these muscles are in an early myoblastic stage at the beginning of this period. By stage N. & F. 40, myofibrils have developed within the constituent cells of the pharyngeal muscles, while in the levator hyoideus, cross-striations are already apparent. Within all of these muscles, nerve-fibres can now be traced, yet none of them as yet show a positive esterase reaction, although in such preparations a longitudinal band of coloured cells can be seen on each side within the hind-brain in the position of the primitive motor tract, both in front of and behind the otic capsule (fig. 1, F).

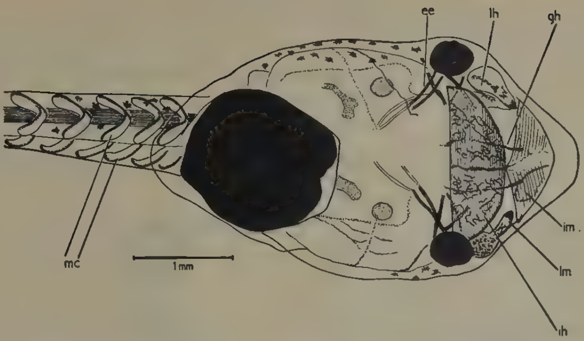

FIG. 5. Esterase preparation of whole larva of *Xenopus* at stage 47. Trunk and part of tail seen from below. *ee*, extrinsic eye-muscles; *gh*, geniohyoideus; *ih*, interhyoideus; *im*, intermandibularis; *lh*, levator hyoideus; *lm*, levator mandibularis; *mc*, tail myocommata.

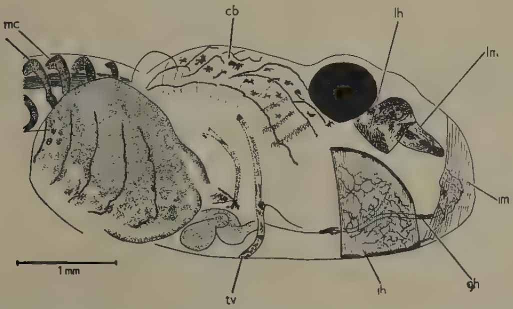

FIG. 6. Lateral view of esterase preparation of *Xenopus* larva at stage 47. The tadpole was divided into two through the median vertical plane. The innervation of the abdominal wall shows through the pigmented coelomic wall. *cb*, constrictor branchialis; *gh*, geniohyoideus; *ih*, interhyoideus; *im*, intermandibularis; *lh*, levator hyoideus; *lm*, levator mandibularis; *mc*, tail myocommata; *tv*, transversus ventralis.

In esterase preparations at stage N. & F. 41, the coloration within the hindbrain is stronger than before, and now for the first time a positive reaction is seen in the external eye-muscles, and in some of the pharyngeal muscles (fig. 7), namely, the levator mandibulae, the levator hyoideus, the geniohyoid, and the interhyoideus. At this stage, the coloration in the latter is confined almost entirely to the lateral margin and to the median raphe. By now motor-fibres have entered the lateral margin of the interhyoideus, and have grown mesially to end in the mid-line on each side. The esterase preparations show that only at their lateral entry into the muscle and at the median termination is the enzyme as yet to be detected.

In both the genio-hyoid and the external muscles of the eye, a positive esterase reaction appears as soon as their respective motor-fibres have entered. At stage N. & F. 40, the external eye-muscles are cross-striated while the genio-hyoids have not yet reached this stage of differentiation. Again, at this stage nerve-fibres have already reached the transverse ventralis muscle, which meets on each side below the bulbus cordis, but this muscle does not become esterase-positive until stage N. & F. 44, some 30 h later, by which time cross-striations are apparent in it. There is thus no uniformity in the order in which the various muscles receive their innervation, become cross-striated, and give a positive esterase (table 1). Concerning the spatial distribution of esterases within the various types one generalization, however, can be made. In long strap-like muscles, such as the external eye-muscles and the geniohyoideus, only a slight terminal concentration of the enzyme is evident, while in broader muscles a positive reaction is at first confined to the ends of the fibres, a

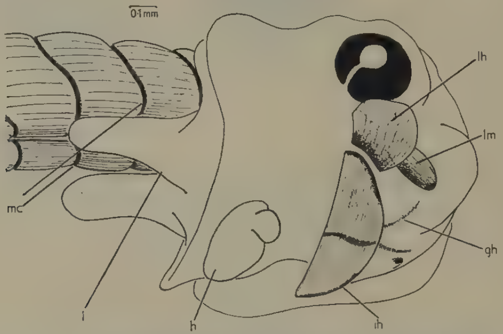

FIG. 7. Esterase preparation of larva of *Xenopus* at stage 41, partly dissected, and seen from ventro-lateral aspect. *gh*, geniohyoideus; *ih*, interhyoideus; *h*, heart; *l*, lungs; *lh*, levator hyoideus; *lm*, levator mandibulae; *mc*, tail myocommata.

TABLE 1

Nieuwkoop and Faber stages

Muscle	*Becomes cross-striated*	*Receives innervation*	*Shows esterase reaction*
Anterior myotomes	32	32	32
External eye-muscles	40	41	41
Interhyoideus	43½	40	41
Levator hyoideus	40	40	41
Levator mandibulae	43	40	41
Geniohyoideus	43½	41	41
Transverse ventralis	43½	41	44
Intermandibularis	46	45	47
Constrictor branchialis	46	44	45
Muscles of abdominal wall	43	43½	45

distribution which recalls its myocommatal location within the axial musculature. The transverse ventralis muscle consists of four bundles of fibres on each side attached end-to-end. The enzyme reaction first appears at these nodal points and subsequently continues more intense there than elsewhere within the muscle.

In thin muscle sheets, such as that of the abdominal wall and the constrictor branchialis, in both of which a positive esterase reaction is first seen at stage N. & F. 45, a single motor-fibre, along which an intense coloration is evident, runs in the septum between adjacent muscle segments. The nerves course in parallel lines within the abdominal muscles, and also in the lower and lateral part of the constrictor branchialis. Over the dorsal surface of the branchial chamber the arrangement of muscle and nerve is less regular, though even here the esterase reaction is at first confined to the ends of the muscle-fibres.

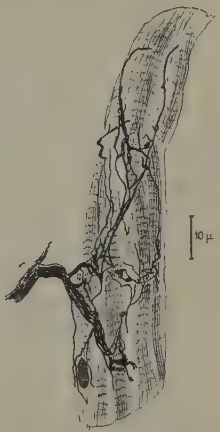

FIG. 8. From a horizontal section through a larva of *Xenopus* at stage 46, to show relatively complex nerve-endings on an extrinsic eye-muscle.

In many muscles the primary innervation is later augmented by another. The constrictor branchialis form a thin sheet, consisting only of a single layer of fibres. The development of the secondary pattern of innervation can here be seen particularly clearly. Motor-nerves spread gradually across the muscle-fibres, and repeated patches of esterase appear along their course (fig. 1, J). From these elements an elaborate tracery is constructed. Again, in the interhyoideus, the primary terminal reaction of the fibres is supplemented within about two days by an intermediate zone of distribution of the enzyme. The motor-fibres which have grown mesially through the muscle acquire new junctions with the muscle-cells, which in esterase preparations are evident as a delicate network between the original sites of reaction (fig. 1, I). Although that within the median raphe remains undiminished in intensity, nerve-fibres in this zone become less conspicuous than elsewhere within the muscle. In general the esterase reaction is most intense at points where muscle-fibres are inserted in the mid-line. In long, narrow muscles, the motor-endings themselves become more complex as development progresses. By stage N. & F. 46, the neuromuscular junctions within the external eye-muscles take the form of a tangled plexus of fibres, some elements of which return upon themselves to form closed loops (fig. 8). Similar configurations are also to be seen within the long genio-hyoids. Thus with respect to their type and distribution of nerve-endings, these muscles stand at one end of a series, the opposite extreme of which is represented by the myotomes.

The muscles of the abdominal wall also acquire a secondary pattern of innervation. This first becomes noticeable in esterase preparations at about stage 48 as occasional isolated groups of endings, usually near the midline (fig. 9, A, B). The secondary pattern spreads quite slowly; a week after its first appearance it is complete only over the most anterior segment (stage 50) and only after a further two weeks is it fully developed over the more posterior segments (about stages 53 to 55). At this stage of development this secondary pattern takes the form of a series of endings along the course of each muscle-fibre (fig. 9, C). Immediately after metamorphosis, however, most of the abdominal muscles have a different secondary pattern in which the endings run in groups obliquely across the muscle-fibres (fig. 9, D) with each muscle-fibre receiving one, or at the most two, endings along its length. The septal esterase activity at the ends of the muscle-fibres is still present (fig. 9, D). This apparent change in the secondary pattern is probably due to the fact (Nieuwkoop and Faber, 1956) that there is a progressive development of new muscles in this region during late tadpole stages with disappearance of some of the original abdominal musculature at metamorphosis.

Discussion

A number of workers have assayed the cholinesterase of developing Amphibia by chemical methods (Youngstrom, 1938; Sawyer, 1943 *a*, *b*; Boell and Shen, 1950). All have used *Amblystoma*; Youngstrom in addition worked on two anuran species. Each of these investigators has demonstrated a rapid rise in the content of the enzyme from the time when the larva begins to swim, though in some points there are differences in detail between their findings. Sawyer found that some cholinesterase was present in the embryo as early as the neurula stage in 'presumptive nerve and muscle'. Boell and Shen, however, found that the 'first appearance of cholinesterase in detectable amounts in the spinal cord coincides with the ability of the embryo to respond neurogenically to tactile stimuli'.

Sawyer (1943*b*) measured cholinesterase in the somatic musculature of larvae from which the spinal cord had been removed. In such myotomes with which no motor nerve-fibres had ever made contact, the enzyme was still found to be present, though in much less amounts than in the normally in-

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FIG. 9 (plate). A, stage 48; part of abdominal muscle stained by the Bodian technique after the esterase procedure, showing the primary reaction at the septum (on the right) and the earliest development of the secondary pattern of innervation from a bundle of nerve-fibres branching off from the septum.

B, a higher power view of part of area reproduced in A showing some of the myo-neural junctions of the developing secondary pattern.

C, stage 55; part of abdominal muscle, showing secondary pattern of motor-endings along course of muscle-fibres.

D, soon after metamorphosis; part of abdominal musculature, showing groups of motor-endings running obliquely across the muscle-segment and persistent esterase activity at the ends of the muscle-fibres.

E, stage 55; part of the lateral segment of the transverse ventralis muscle, showing the profuse pattern of motor-endings.

F, a higher power view of part of the same muscle-segment reproduced in E.

G, stage 48; part of the lateral segment of the transverse ventralis muscle stained by the Bodian technique after the esterase procedure, showing a single nerve-fibre forming 5 esterase-positive junctions (arrowed) with three muscle-fibres over a total length of about 150 μ.

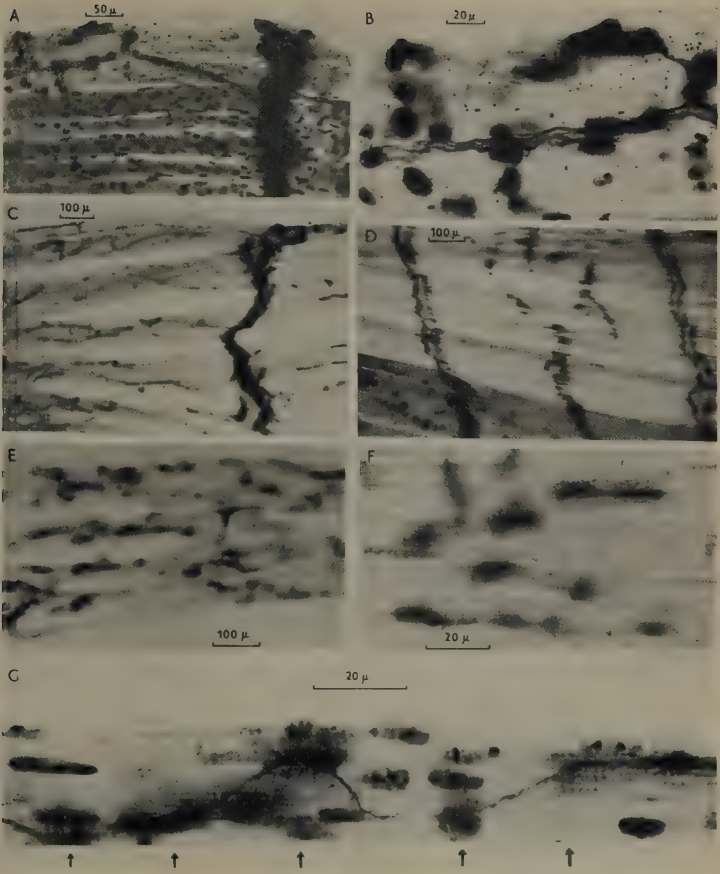

FIG. 9

P. R. LEWIS *and* A. F. W. HUGHES

nervated muscle of the corresponding age. Sawyer's result thus indicates that the cholinesterase of the axial musculature of *Amblystoma* has a dual origin, partly intrinsic, but with the greater part originating in some way through contact with motor nerve-roots.

In the embryo of *Xenopus* the first signs of a positive esterase reaction is in the anterior region of the spinal cord at stage N. & F. 28. A myocommatal reaction beyond the cord is first seen in the succeeding stage. The correlation of these physiological events with the development of the spinal motor-roots is hampered by the fact that nerve-fibres at these stages have as yet no affinity for silver, either in *Xenopus* or in *Amblystoma*, where Coghill (1913) found that impregnation methods were not of assistance until the early swimming stage had been reached.

However, our observations on all other muscles which develop subsequently to the axial musculature have shown that motor-nerves arrive either before or at the same time as an esterase reaction first appears. Thus a group of pharyngeal muscles which are innervated at stage N. & F. 40 show a positive esterase reaction in the following stage. The cell-bodies of their motor-fibres within the motor tract of the hind-brain are first esterase-positive at stage N. & F. 40, from which time the reaction increases continuously in intensity. Here, then, the evidence points clearly to the interpretation that the enzyme, or some precursor, is synthesized within the perikaryon, and reaches the nerve-muscle junction by efferent flow along the axon (Lewis and Hughes, 1957).

Both in the axial musculature and elsewhere there is a close correlation between the first appearance of cholinesterase and the onset of reflex behaviour in each muscle. This correlation was particularly striking in a tadpole at stage N. & F. 29, which responded to stimulation by a simple flexure of the forepart of the trunk, and was found to show enzyme activity in only a few anterior myocommata. In the pharyngeal musculature, enzyme activity and reflex behaviour develop simultaneously, for when the enzyme is first detectable the response of the muscle is rudimentary, and co-ordinated movement only begins at about the stage when the pattern of the distribution of the enzyme approaches completion.

Very different patterns of innervation are seen in the various muscles and it is possible to suggest relationships between each particular arrangement and the type of activity demanded of the muscle. In the transverse ventralis muscle, for instance, each lateral segment consists only of a few muscle-fibres of relatively small diameter, in which an enormous number of separate patches of esterase activity are seen (fig. 9, E, F). A study of segments dissected from esterase preparations and then stained by the Bodian technique showed that each of these patches of esterase activity was a genuine myo-neural junction, though simple in form. They are distributed along almost the whole length of each muscle-fibre at intervals of 30 to 150 μ (fig. 9, G), a branch of the same nerve sometimes innervating several consecutive junctions on the same muscle-fibre. Although it was not possible to make an accurate count of both muscle-fibres and myo-neural junctions in a single segment, rough counts suggest that each muscle-fibre possesses some 10 to 20 junctions along its whole length. It seems likely that this pattern is in fact that of the 'slow muscle innervation' known to be present in amphibians (Kuffler and Vaughan Williams, 1953). These slow muscle-fibres have a multiple innervation which does not cause a twitch but only a slow contracture which is induced by electrotonic spread of depolarization from the individual myo-neural junctions. A similar pattern of innervation is seen in some of the abdominal muscles before metamorphosis (fig. 9, C). The muscle-fibres of the constrictor branchialis also have a multiple innervation though here the nerve-fibres run mainly across the muscle-fibres (fig. 1, J).

In the extra-ocular muscles the profuse distribution of separate motor end-plates may be correlated with the need for precisely-graded reflex responses in these muscles. At an opposite extreme is the pattern seen in the main axial musculature, where each muscle-fibre is innervated only at its ends from plexuses in the myocommata (fig. 1, B). This innervation of the myotomes calls for some comment since it is so unlike the usual type of myo-neural junction in vertebrate muscle. Myocommatal endings of nerve-fibres in lower vertebrates have previously been described by Giacomimi (1898 *a*, *b*), who, however, regarded them as wholly sensory in nature. In the early development of the motor system in *Amblystoma*, Coghill (1926, pp. 101, 102) recognized that some branches of spinal motor-nerves end at the myocommata. Again, in Harrison's classical paper (Harrison, 1924) on the respective contributions of neural crest and cord to the development of the peripheral nervous system in this urodele, he showed that the first motor-fibres to the abdominal musculature end at the septal planes, while the sensory nerves spread generally over the surface of the segmental units. Terminal motor-endings to muscle-fibres in selachians have recently been described by Couteaux (1955). Although the existence of myo-neural junctions at both ends of the muscle-fibre would not seem to be a suitable general method of obtaining well co-ordinated reflex activity, it might have a specific advantage in the larva of *Xenopus*, which passes through the early stages of development at great speed; a sharp avoiding-reaction to tactile stimulation appears within considerably less than 48 h of fertilization. Nerve-fibres are believed to grow most readily along surfaces (contact guidance; Weiss, 1941) and hence functional contact between the motor nerve-fibres and the myotomal muscles might be most rapidly achieved along the myocommatal planes—i.e. at the ends of the muscle-fibres. Some observations on the regenerating tail-bud of the lizard, *Sphaerodactylus*, are of particular relevance here (Hughes and New, 1959). The muscles in the normal tail of this lizard have large classical end-plates along the course of each muscle-fibre. When muscle-fibres first develop in the regenerating tail, however, they are first innervated only at their ends and give a pattern reminiscent of that of the myotomes in *Xenopus* tadpoles. At a later stage the nerve-fibres grow out over the surface of the muscle to form the normal vertebrate type of end-plate. Innervation at the ends of the muscle-fibres may thus be a special adaptation to permit of the earliest possible neurogenic activity, where speed of development is important and accurate control of response is not. This idea might also explain the first appearance of enzyme activity at tendinous septa in several muscles, e.g. interhyoideus, the abdominal muscles, &c., where the more normal type of myo-neural junction along the length of each muscle-fibre is formed later, when presumably the need for co-ordinated reflex activity arises.

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The Nissl–Golgi Complex in the Purkinje Cells of the Tawny Owl, *Strix aluco*

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SUMMARY

The purpose of this investigation was to repeat as exactly as possible the original work on the 'apparatus' of Golgi in the Purkinje cells of the cerebellum of owls, and to re-investigate these cells by modern methods. The tawny owl, *Strix aluco*, was used instead of the closely-related '*Strix flammea*' of Golgi. Golgi's 'osmio-bichromique' technique of 1898 for silver impregnation was used successfully.

A reticulum corresponding to the basiphil *Netz* of Nissl can be seen in the living neurones by interference microscopy. The classical Golgi apparatus of the perikaryon is a deposit of silver or of osmium on this reticulum. The reticulum extends into the axons in the form of thin non-basiphil filaments, which are also blackened by Golgi methods.

These findings are in conformity with recent studies of the neurones of other vertebrates.

INTRODUCTION

IN a recent publication (1959) I showed that a three-dimensional reticulum could be seen by interference microscopy in the living neurones of vertebrates. This reticulum was homologized with the basiphil reticulum (*Netz*) described by Nissl (1894 *a*) in these cells. Evidence was presented to show that the classical 'Golgi apparatus' in the perikaryon of the neurone of vertebrates was a deposit of silver or of osmium on this basiphil reticulum. In the axon, however, the 'Golgi apparatus' consists of non-basiphil filaments, which are extensions of the basiphil reticulum.

It was my purpose to extend this study to the site in which Golgi first described his 'apparatus', namely, the Purkinje cells of the cerebellum of owls. Golgi (1898) called his owls *Strix flammea*, by which he probably meant the barn-owl, *Tyto alba*. I have not been able to obtain this species, and have used instead the closely related tawny owl, *Strix aluco* (L.).

It would not appear that anyone else has examined the Purkinje cells of owls in the 60 years that have elapsed between Golgi's investigations and my own.

MATERIAL AND METHODS

Two live tawny owls were obtained from Mr. Robert Jackson, Holly Bank Nurseries, Grove Lane, Hale, Cheshire. These were killed by chloroform and the cerebellum was at once dissected out.

Freshly teased Purkinje cells mounted in saline with calcium chloride (Baker, 1944) were studied by positive phase-contrast and with the Baker interference microscope (× 100 double-focus objective).

[Quarterly Journal of Microscopical Science, Vol. 101, part 1, pp. 69-74, March 1960.]

Sections of material fixed in formaldehyde-calcium and postchromed (Baker, 1946) were coloured by Sudan black (Baker, 1949) and acid haematein (Baker, 1946) for the study of lipid inclusions.

Silver and osmium preparations were made by the methods of Aoyama (1929) and Mann-Kopsch (Weigl, 1910) respectively.

The greater part of the available material was used up in making preparations by Golgi's original method, the 'osmio-bichromique' technique of 1898, which would not appear to have been tried by anyone during the last half century. Small pieces of the cerebellum were fixed in a mixture of 2 volumes of 3% aqueous solution of potassium dichromate and 1 volume of 1% osmium tetroxide. Pieces were taken out of this fluid at intervals of 1, 2, and 3 months, directly transferred to a 3% aqueous solution of copper acetate (for 'rejuvenation'), and left for 2 or 3 days. The pieces were subsequently passed into a 3% aqueous solution of potassium dichromate. In accordance with the suggestion of Golgi, repeated trials were made with different periods, from 3 days to a fortnight, in this fluid. Details of the after-treatment of these pieces were taken from an earlier paper by Golgi (1886). Golgi presumably cut sections by hand. Since this could not be done with the small pieces I used, sections were mostly cut by embedding in collodion (low viscosity nitrocellulose).

When the pieces are transferred from potassium dichromate to silver nitrate, a precipitate of silver chromate is formed (Golgi, 1886). A few preliminary washings were, therefore, given in a 0·5% solution of silver nitrate till no more precipitate appeared, and the tissue was then left in a 0·75% solution of silver nitrate for 24 to 48 h in daylight. (According to Golgi (1886), it makes no difference whether the tissue is left in the light or not.)

The best Golgi pictures were obtained in tissue fixed for 2 months, rejuvenated for 2 days, and treated with potassium dichromate for about a week. Cellular outlines were not very conspicuous in these preparations. They were better seen in sections of tissue fixed for 3 months, rejuvenated for 3 days, and treated with potassium dichromate for 4 to 6 days.

After silvering, the material was washed in several changes of 70% or 95% alcohol, and passed through absolute alcohol into a mixture of equal volumes of absolute alcohol and ether, and thence to collodion. The sections were cut at 15 or 20μ. Frozen and paraffin sections were also made, but the results were not satisfactory.

Collodion sections were dehydrated in 95% alcohol and creosote and cleared in turpentine. They were mounted in dammar (in xylene), with or without coverglass. If the collodion is removed from the sections by soaking in absolute alcohol, they break up; it was, therefore, not removed.

Nissl bodies were shown by bleaching Mann-Kopsch or Aoyama preparations (see Malhotra, 1959) and staining in pyronine / methyl green (Jordan and Baker, 1955) or in cresyl violet (Fernstrom, 1958). Alternatively, pieces of cerebellum were fixed in Mann's fluid with the addition of acetic acid (Baker, 1957), and stained in the same ways.

Sections of material fixed in Palade's buffered osmium (Palade, 1952) and embedded in *n*-butyl methacrylate were cut at about 3μ, mounted in *n*-butyl methacrylate, and studied unstained by phase-contrast.

In an attempt to show the mitochondria of the Purkinje cell, pieces of cerebellum were fixed in Helly (1903) and postchromed; sections were stained by Metzner's and Hirschler's methods.

RESULTS

In living Purkinje cells studied by interference microscopy, a prominent reticulum can be clearly seen in the perikaryon. This reticulum, as described already in the neurones of other vertebrates (Malhotra, 1959), consists of massive bodies of varying sizes and shapes scattered throughout the cytoplasm, except at its extreme periphery. They are bigger and more concentrated round the nucleus. They do not seem to be sharply delimited from the ground cytoplasm. These massive bodies are connected with one another by thin strands, which show the same interference colour as the massive bodies. The strands may be straight or loop-shaped. There are also conspicuous, optically homogeneous spaces of very low refractive index in association with both the components of the reticulum. These 'canalicular spaces' are either elongate or irregularly rounded. They are bigger and more easily detectable than in the neurones of the mouse (Malhotra, 1959).

Apart from this reticulum, numerous small refringent globules (presumably lipid globules) are seen by interference microscopy, dispersed at random throughout the cytoplasm of the living Purkinje cells.

These refringent globules are better seen in living cells by phase-contrast microscopy. The canalicular spaces are also sharply outlined by phase-contrast. There are some indications of the presence of the strands of the reticulum, but the massive bodies cannot be seen in living neurones by phase-contrast.

All the structures described in the above paragraphs, except the massive bodies, can easily be seen in unstained sections of tissue that has been fixed in Palade's buffered osmium and examined by phase-contrast. The massive bodies cannot be very clearly demonstrated in these preparations, probably because they are not sharply delimited. Small dark globules, which look like the lipid droplets, often appear to lie in short rows on the strands of the reticulum, but they are too small for reliable observation.

In Nissl preparations of the Purkinje cells, the basiphil component is found to exist in the form of large, irregular bodies of varying sizes and shapes (commonly called Nissl bodies), distributed in the cytoplasm except at its extreme periphery and in the axon hillock. They are bigger and sometimes more elongated round the nucleus. There are thread-like basiphil connections (*Fäden* of Nissl, 1894 *b*) joining these Nissl bodies with one another to form a reticulum (*Netz* of Nissl, 1894 *a*). There are also canalicular spaces in association with the basiphil threads and with the Nissl bodies (fig. 1, B). This pattern of the basiphil reticulum agrees essentially with the reticulum seen in the living neurone by interference microscopy and also with the structures seen in unstained sections studied by phase-contrast. It is clear that the reticulum that is discernible in life by interference microscopy corresponds to the basiphil reticulum of routine Nissl preparations.

In Golgi preparations made by the method of Aoyama, the Golgi apparatus has a characteristic disposition, resembling that of the basiphil reticulum. The canalicular spaces are generally not seen in Aoyama preparations, presumably because they have been covered by a deposit of silver. If, however, these sections are bleached and studied after staining in basic dyes (Malhotra, 1959), the existence of these spaces is easily established. These spaces, covered with silver, account for some of the massive thickenings in Golgi preparations.

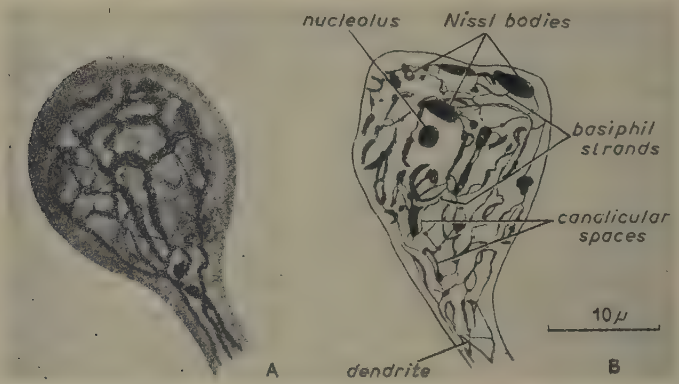

FIG. 1. A, Purkinje cell from '*Strix flammea*', reproduced from Golgi (1898). This is the earliest figure of Golgi's 'apparatus'. 'Osmio-bichromique' method. It is not certain whether the process in this figure is an axon or a dendrite. B, Purkinje cell from *Strix aluco*, showing basiphil reticulum in the perikaryon. Aoyama (desilvered), pyronine / methyl green. The nuclear membrane is covered by Nissl bodies, and is not clearly seen.

In Mann–Kopsch preparations (4 days' osmication at 34° C) the Golgi pictures are mostly filamentous; the canalicular spaces are easily seen, associated with osmiophil threads. Large blackened lumps resembling Nissl bodies also occur in a few cells.

Both in silver and in osmium preparations the Golgi apparatus is sometimes seen to extend into the axon in the form of extremely fine filaments. There are often what appear to be lipid globules lying in rows on these filaments. More often than not the axon hillock appears to be empty in Golgi preparations. This led many cytologists of the past to believe that the Golgi apparatus does not extend into the axon (see Malhotra, 1959, for references); but Gatenby and his colleagues (1949, 1953) have also recently recorded the continuation of the Golgi apparatus into the axon of the neurones of vertebrates.

Suitable preparations made by the silver impregnation method of Golgi (1898; see p. 70) reveal that the Golgi picture resembles those produced by

Aoyama's method, and also resembles Golgi's own figure (1898) depicting his 'apparatus' in the Purkinje cells of '*Strix flammea*' (fig. 1, A). Sometimes, however, the impression is given that the dark objects are only a random deposit of silver, lying superficially in the cell. The nucleus and the cytoplasm of the periphery of the cell are almost always free from this impregnation. It seems unlikely, therefore, that the black material in these preparations is actually a random deposit of silver. It seems more likely that the initial deposit of silver is on the basiphil reticulum.

In Sudan black preparations, small lipid globules are seen scattered in the cytoplasm. They correspond in size and distribution to the refringent globules seen in living neurones by phase-contrast or interference microscopy. Similar globules react positively to the acid haematein test, which also darkens a reticular structure in some of the Purkinje cells. This structure appears to be the basiphil reticulum. It is remarkable that no reticulum has been seen in acid haematein preparations of the neurones of other vertebrates (Casselman and Baker, 1955; Malhotra, 1959). The reticulum of the Purkinje cell of the owl has an unusually great affinity for silver and osmium, and this fact may perhaps be correlated with a large phospholipid component, or with a phospholipid component that is easily 'unmasked'. This may have been the reason why Golgi discovered his 'apparatus' in this particular cell of owls. The reliability of acid haematein test for phospholipid was not checked by pyridine extraction test (Baker, 1946), on account of lack of material.

No satisfactory mitochondrial preparations could be made either by Metzner's or by Hirschler's method of staining, because in these sections a reticular structure (which is almost certainly the basiphil reticulum) is stained and the mitochondria cannot be properly differentiated. This again suggests the presence of phospholipid in association with the reticulum.

I am greatly indebted to Dr. J. R. Baker, F.R.S., for supervising this work and for very helpful criticism, and to Professor Sir A. C. Hardy, F.R.S., in whose department the work was carried out. I thank Dr. Leonard Harrison Matthews, F.R.S., of the Zoological Society of London, for making this investigation possible by putting me in touch with a dealer who possessed tawny owls.

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The Cytoplasmic Inclusions of the Neurones of Crustacea

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With one plate (fig. 2)

SUMMARY

Four kinds of cytoplasmic inclusions can be recognized in the neurones of *Leander serratus* and *Astacus fluviatilis*. These are (i) spherical or almost spherical bodies, which often show a differentiated cortex and medulla; (ii) mitochondria, in the form of minute granules and short rods; (iii) Nissl substance, uniformly dispersed; (iv) 'trophospongial' structures, which are connected with the surface of the cell, and ramify in the form of delicate filaments throughout the cytoplasm.

Neutral red colours the spherical bodies in life; it does not seem to interfere with their optically visible structure. The spherical bodies often burst open into rods and crescents; these correspond to what other authors have called 'Golgi apparatus' or 'dictyosomes'. The term 'Golgi apparatus' has also been applied by certain authors to the 'trophospongial' structures.

Histochemical study reveals that the surfaces of the spherical bodies, which are blackened by osmium tetroxide or silver nitrate in the Golgi methods, respond to tests for phospholipid after an 'unmasking' fixative has been used. The evidence also suggests the presence of cerebroside (galactolipid) in these bodies.

THOSE who have studied the neurones of Crustacea have given very divergent accounts of the 'Golgi apparatus' of these cells. Poluszynski (1911) described only short, straight or slightly sickle-shaped threads; he considered them as the Golgi apparatus. Monti (1915) believed that the Golgi apparatus of this cell was constituted by the mitochondria. She thought that they were long, wavy threads, which were blackened by the Golgi methods and formed a reticulum of the type seen in the neurone of vertebrates. Ross (1922) described a framework of 'trophospongium' connected with the surface of the cell. He thought this was additional to a reticular 'Golgi apparatus'.

In view of these and other diverse opinions on the Golgi apparatus, I thought it desirable to reinvestigate the cytoplasmic inclusions of this cell and to try to interpret the above findings in the light of my own results.

MATERIAL AND METHODS

Neurones from the thoracic and abdominal ganglia of the prawn, *Leander serratus*, and the crayfish, *Astacus fluviatilis*, were investigated. The prawns were obtained from the Marine Biological Laboratory, Plymouth, and kept until wanted in an aquarium. They were fed on *Mytilus*. The crayfish were obtained from the Surrey Trout Farm, Haslemere, Surrey. They were also kept in an aquarium and fed on cod.

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Methods

1. Phase-contrast and polarizing microscopy for the study of unstained, living neurones.

2. *Vital dyes.* Neutral red, dahlia, and Janus green. Two drops of a 0·5% aqueous stock solution diluted with 2·5 ml of sea-water for the neurones of *Leander*.

3. *Buffered osmium.* Tissue was fixed in buffered osmium (Palade, 1952) or buffered osmium with the addition of calcium chloride (Baker, 1958) and embedded in *n*-butyl methacrylate as for electron microscopy. Sections were cut at about 3 μ and mounted in the monomer or in glycerol (Baker, 1960); these were studied by phase-contrast or interference microscopy.

4. *Mitochondrial techniques.* Material fixed in Helly (1903) for about 24 h (with postchroming) or Altmann or 'NH_4-Altmann' (Baker and Luke, 1959) was stained by the method of Hirschler (1927) or Metzner (1928).

5. *Nissl techniques.* Tissue fixed in Zenker (1894; 3 h) or Helly was stained in pyronine / methyl green (Jordan and Baker, 1955) or cresyl violet (Fernstrom, 1958) or basic fuchsine. Ribonuclease (Bradbury, 1956) and trichloroacetic acid (Pearse, 1954) were used for control.

6. *Golgi techniques.* The osmium impregnation methods of Kopsch (1902), Mann-Kopsch (Weigl, 1910), and Kolatchev (1916; after fixation in Champy, 1911) were used. The Mann -Kopsch method was also used on neurones that had previously been vitally coloured with neutral red. The colouring of the neurones was checked before transferring the tissue to the fixative. Post-osmication lasted 24 h in this case.

Ganglia of *Leander* were also fixed in a solution of osmium tetroxide in sea-water and post-osmicated as usual. When this material is embedded in paraffin, the results are not satisfactory. If, however, the tissue is embedded in methacrylate, the finished preparations are good. The details of this technique are as follows.

(i) Fix in a 1% solution of osmium tetroxide in sea-water (1 ml of a 2% aqueous osmium tetroxide and 1 ml of sea-water with the addition of 3·5 g of sodium chloride per 100 ml) for 2 to 3 h. Fixation for longer periods is not satisfactory.

(ii) Wash thoroughly in running water to get rid of the salts present in the sea-water; give a last wash in distilled water.

(iii) Post-osmicate in a 2% aqueous solution of osmium tetroxide at 34° C for 3 to 4 days.

(iv) Wash in running water for 6 h.

(v) Dehydrate and embed in *n*-butyl methacrylate.

(vi) Cut 3 μ sections and attach them to the slides like paraffin sections (Baker, 1960).

(vii) Dissolve the polymer in ethyl acetate.

(viii) Mount in Canada balsam.

Silver impregnation methods of Golgi (1898, dichromate/osmium; 1908, arsenious acid), Veratti (Golgi, 1900), Ramón y Cajal (1914), and Aoyama (1929) were used.

7. Material fixed in Helly, Lewitsky–saline (Baker, 1956*a*), and Carnoy (1886) was stained in iron haematoxylin.

8. Rawitz's 'inversion staining' technique (Baker, 1959; Przełęcka, 1959).

9. Tissue fixed in Helly and embedded in paraffin was stained with Masson's tricolor stain (Pantin, 1948) or xylidine red alone.

10. Tissue fixed in Zenker and embedded in paraffin was stained with Mallory's phosphomolybdic acid / haematoxylin (1938).

The details of the techniques used for histochemical investigation of the neurones of *Leander* are given in the appendix. The neurones of *Astacus* were not studied histochemically.

RESULTS

Most of the previous work has been done on the neurones of *Astacus fluviatilis*. I hope to repeat these observations as far as possible. But these cells are difficult to fix well, especially the large cells. This difficulty of fixation has also been experienced by other workers on the neurones of Crustacea (Monti, 1915; Ross, 1922). For this reason I extended my study to the neurones of the prawn, *Leander serratus*; and I found them rather convenient for this investigation.

The cytoplasmic inclusions of the neurones of *Leander* will first be considered in detail. The differences shown by those of *Astacus* will then be mentioned.

Morphology

Neurone of Leander

A wide variation occurs in the size of the nerve-cells in a ganglion. They can conveniently be grouped into small, medium-sized, and large neurones. The cytoplasmic inclusions discussed below are very similar in all three categories of cells, and a single description will suffice.

It will be helpful to the reader to start by mentioning briefly the kinds of cytoplasmic inclusions present in this cell, and then to relate in turn the appearances seen after the application of particular techniques.

Fig. 1 provides a summary of the results obtained. Four kinds of cytoplasmic constituents can be demonstrated. These are:

(1) Spherical or almost spherical bodies, which often show a binary structure; that is to say, the surface is distinct from the interior.

(2) Mitochondria in the form of short rods and granules.

(3) Nissl substance, diffusely distributed.

(4) Hollow tape-like objects, which penetrate into the neurone from its surface, and ramify in the form of filaments in the cytoplasm. These correspond to the 'trophospongium' of Ross (1922). They appear to be the invaginations of the cell membrane. The contents of the cells surrounding the neurone flow into these invaginations.

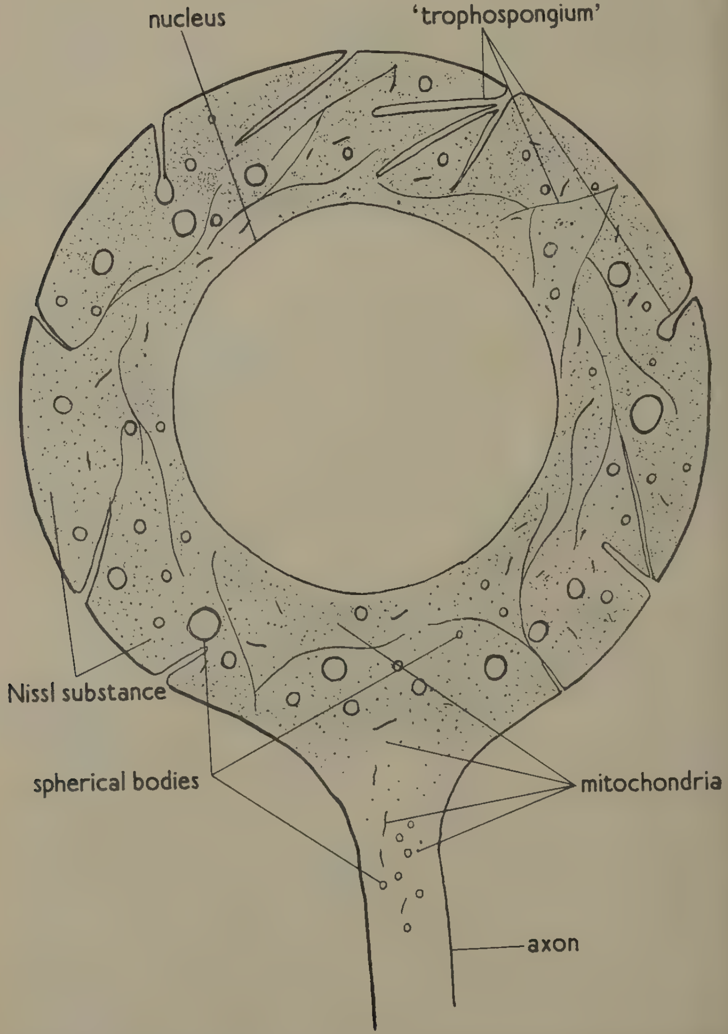

FIG. 1. Diagrammatic illustration of the cytoplasmic inclusions of the neurone of *Leander*.

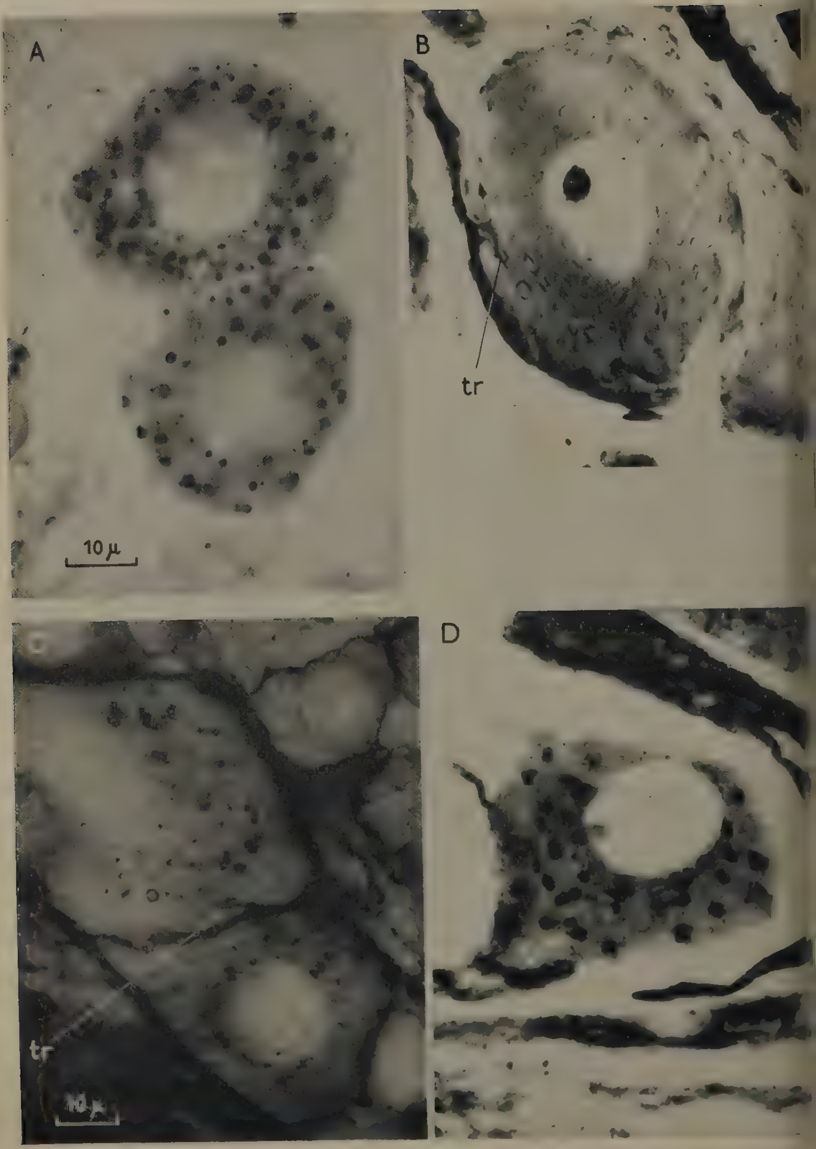

FIG. 2

S. K. MALHOTRA

In the living neurone studied by phase-contrast microscopy, refringent spherical or almost spherical bodies are the most prominent of the cytoplasmic constituents. They are dispersed at random. They vary considerably in size up to about 2 or $2\frac{1}{2}$ μ in diameter. These spherical bodies often show a dark surface and a pale interior. This appearance suggests that lipid is probably localized at the surface. At times the dark edge seems to be unevenly spread round the pale globules. They seem to be very delicate structures, as they tend to become distorted under the pressure of the coverslip. These refringent spheres also extend into the axon, but here they are small and optically homogeneous.

In addition, phase-contrast reveals in the living neurone long, wavy, filamentous structures throughout the cytoplasm. These are very thin and are not always clearly seen. Sometimes tape-like structures which appear like invaginations from the cell membrane are also seen.

No pigment is seen in the living neurone.

The cytoplasmic objects that are coloured with neutral red, while the cell is still alive, correspond in size and distribution with the spherical bodies observed in the unstained neurone by phase-contrast. When such cells are examined by direct microscopy the bodies dyed with neutral red appear homogeneous (fig. 2, A). Sometimes the impression is given that the neutral red globules are more intensely coloured at the periphery than internally. If these preparations are studied by phase-contrast microscopy, most of the neutral red globules show a dark surface, such as is seen in the unstained cell. This shows that neutral red does not interfere with the optically visible structure of the bodies that are vitally dyed by it. Small globules coloured with neutral red are also seen in the axon.

Living neurones dyed in Janus green and studied by direct microscopy show minute granules, granules lying in small rows, and small rods (fig. 3). These are presumably mitochondria. Janus green does not colour the very long filamentous structures seen in the unstained cell by phase-contrast; they do not appear to be mitochondria.

Dahlia does not colour any of the cytoplasmic inclusions visible in the living cell by phase-contrast; the dye is uniformly dispersed in the ground cytoplasm.

Thin sections of ganglia fixed in buffered osmium (Palade, 1952) and studied by phase-contrast (Baker, 1960) do not generally show the spherical bodies that are so obvious in the living cell. Instead, objects shaped like the letter U and V, and like bananas and crescents are seen. Even rings are also

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FIG. 2 (plate). A, neurone of *Leander* vitally dyed with neutral red.

B, neurone of *Leander*; Altmann/Metzner; note chromophil crescents associated with chromophobe material, and also 'trophospongial' prolongation (tr) from the surface of the cell into the cytoplasm of the neurone.

C, neurone of *Leander*; Aoyama (with postchroming) / acetylated Sudan black; note the binary structure of the sudanophil spherical bodies (life-like), and 'trophospongium' (tr).

D, neurone of *Astacus*; Kolatchev.

The scale on fig. C also applies to B and D.

found, but they are very rare. Each of these bodies shows the double structure seen in the spherical bodies by phase-contrast, that is to say, a dark sheath associated with a much lighter area. The latter is often greatly distorted. These are the appearances that are commonly called 'Golgi apparatus' or 'dictyosomes' in the neurones of invertebrates (Thomas, 1947; Moussa, 1950; Beams and others, 1953; Shafiq, 1953, 1954; Young, 1953, 1956; Gresson and others, 1956; Malhotra, 1956, 1957; Chou, 1957 *a*, *b*; also see Lacy, 1957, and Baker, 1959 for references). Sometimes small, dark, curved rods or almost straight rods are also seen in such preparations. These do not show any material associated with them and appear homogeneous under phase-contrast. If, however, these sections are examined by interference microscopy, a thin layer of some material having a very low refractive index is discernible in association with the objects, which appeared homogeneous under phase-contrast. It is conceivable that the material in association with these bodies was originally bigger and was disrupted during the preparation of the tissue, leaving behind its traces which could not always be seen by phase-contrast.

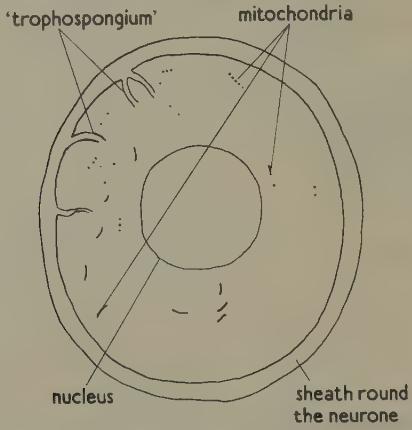

FIG. 3. Neurone of *Leander* vitally dyed with Janus green. The 'trophospongium' is not coloured.

Neurones fixed in buffered osmium and studied by phase-contrast often also show long, slightly wavy or straight objects, which look like narrow tapes, at or near the surface of the cell, where they are connected with the cell membrane. In the interior of the cell thin, delicate filaments are seen. The latter belong to the same category of objects as the long, fine filaments seen in the living cell by phase-contrast. These filaments are in fact the ramifications of the tapes but no connexions between the two objects have been observed in these preparations.

None of the silver impregnation methods has given satisfactory results. Sometimes, however, I have seen long, blackened filaments in the medium-sized neurones after Ramón y Cajal's method (fig. 4, A). It is highly unlikely that these filaments are mitochondria. They do not conform to the description of the objects that are vitally dyed by Janus green (see p. 79), nor do they resemble the objects believed to be mitochondria in fixed preparations (see p. 83). These are more likely to be parts of the framework formed by the branching of the tape-like structures.

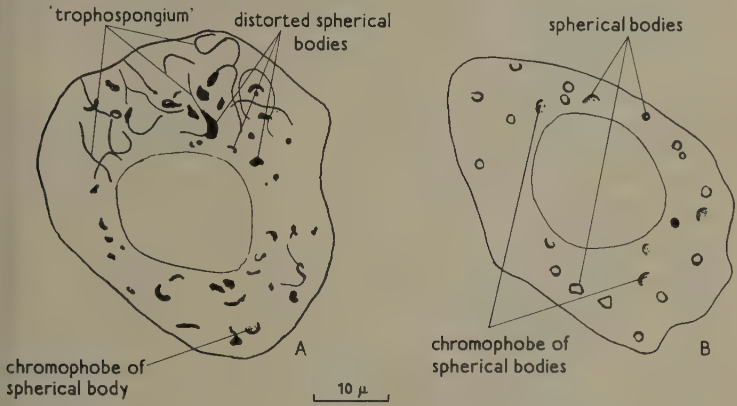
'trophospongium'
distorted spherical bodies
chromophobe of spherical body
A
10 μ
spherical bodies
chromophobe of spherical bodies
B

FIG. 4. Neurones of *Leander* from Ramón y Cajal preparations.

Another kind of cytoplasmic inclusion can sometimes be made out in favourable Ramón y Cajal preparations. This is constituted by the spherical bodies. Some of these are very badly distorted, while the others are in the form of crescents, as seen in buffered osmium neurones. The latter do not always show a chromophobe interior. The spherical bodies are also seen to almost retain the shape they had in life (fig. 4, B); but cells showing such bodies are extremely rare. Some of these argentophil bodies lie in contact with the blackened filaments described in the above paragraph; they give the impression of being enlarged localized regions on the filaments (fig. 4, A). There is no evidence that this is a genuine relationship. It seems to be the result of fixation.

In Mann–Kopsch preparations the objects which readily reduce osmium tetroxide correspond to those seen in the material fixed in buffered osmium and studied by phase-contrast. They are better demonstrated here than in the corresponding silver preparations. Osmiophil material is localized at the surface of the osmiophil sphere. But very often the osmiophil material is seen as large saucers or bananas with the osmiophobe material spread along its length. Such objects measure up to about 7 μ in length. When the osmiophil bodies are ring-shaped (in optical section) they measure up to 3·5 μ in diameter. These osmiophil bodies conform in distribution and binary structure with the spherical bodies seen in the unstained neurone, or one vitally dyed by neutral red; but the biggest of these do not exceed 2·5 μ. The Mann–Kopsch method presumably swells them. A similar swelling of similar bodies has been described by myself (1956) in the neurones of *Schistocerca gregaria.*

The osmiophil bodies are smaller in neurones that had been vitally dyed in neutral red and then subjected to the Mann–Kopsch method. The osmiophil material in these cells also is located at the surface of the spherical bodies, as in routine Mann–Kopsch preparations. This experiment shows that neutral red does not interfere with the osmiophil material of these objects. It will be remembered that a similar sheath could be seen at the surface of the globules dyed with neutral red and studied by phase-contrast (see p. 79).

Spherical bodies that retain their life-like form are rarely met with after Mann–Kopsch treatment. Such objects are, however, common in the neurones that had been fixed in osmium tetroxide in sea-water and then post-osmicated (p. 76). This experiment indicates that the spherical bodies, which often burst open into crescents after fixation in mercuric chloride / osmium, remain stable when osmium tetroxide in sea-water is substituted as the fixative.

The small spherical bodies are blackened all through after post-osmication. Such globules are again more often seen in the neurones fixed in osmium tetroxide in sea-water and post-osmicated than by the Mann–Kopsch method. Fixation in osmium tetroxide in sea-water followed by post-osmication is best of all the 'Golgi' impregnation techniques used on the neurones of *Leander.* The objects that are responsible for the 'Golgi' appearances, that is to say, the neutral red-staining spherical bodies, are preserved in a more life-like form than by other 'Golgi' methods. The darkening of the general cytoplasm is also much less than by the Mann–Kopsch method.

If pieces of ganglia that had been in a 2% solution of osmium tetroxide for about 12 h (according to Kopsch's method) are teased in glycerol and studied by phase-contrast, the spherical bodies of the living cell are represented by bright spheres having a thin dark sheath at the surface. Some of these are distorted and look like bright curved or straight objects with a dark edge. Some of the tissue was left in osmium tetroxide for about a fortnight and studied after sectioning. Such neurones reveal objects similar to those seen in Mann–Kopsch preparations, but the spherical or almost spherical bodies seen after short osmication are often not seen. These seem to have opened up into rods and crescents with or without the remnants of the chromophobe sphere. Neurones prepared in this way are very much shrunk and the osmiophil material is only slightly darkened.

The tape-like structures are also sometimes seen in osmium tetroxide preparations, but they are much less darkened than the spherical bodies. The ultimate branching of these tape-like objects is not clear in these preparations.

The objects described in osmium preparations are very clearly demonstrated in Rawitz preparations. In properly differentiated cells the chromophil component is intensely coloured while the chromophobe sphere is light pink. Sometimes there is little differentiation into these two parts, presumably owing to insufficient differentiation. These preparations also clearly show narrow tape-like structures extending from the surface of the cell into the cytoplasm. These are also intensely coloured by basic fuchsine (after mordanting in tannic acid and potassium antimony tartrate). In this they resemble the surface of the spherical bodies, which are now mostly in the form of crescents and rods. The elongate structures appear to be hollow, though they are tape-like near their attachment to the surface of the cell.

Both the kinds of objects described in the above paragraph are coloured alike by acid fuchsine (Metzner) or alcoholic haematoxylin (Hirschler) or iron-haematoxylin after fixation in Helly (with postchroming) or Altmann (fig. 2, B). When the elongate structures are in contact with the cell surface there is no difficulty in recognizing their true identity. But if the section does not happen to pass through this region they can easily be mistaken for the long filamentous mitochondria. I have been able to study these structures very clearly in neurones fixed in Helly (with post-chroming) and stained in iron haematoxylin (fig. 5, A, B). In suitable sections the branching of these elongate tape-like structures and their ramification can easily be followed. The branching is scarce; and the ultimate branches appear to be thin filaments at the limit of resolution of the ordinary microscope (fig. 5, A), but they may in fact be canalicular like those at the surface of the cell. Ross (1915, 1922) has also described similar delicate threads as parts of the 'trophospongium' forming a framework in the cytoplasm of the neurones of *Cambarus*. A few long, delicate filaments corresponding to these fine branches have also been seen in favourable preparations of neurones fixed in Lewitsky-saline and stained in iron haematoxylin (fig. 6).

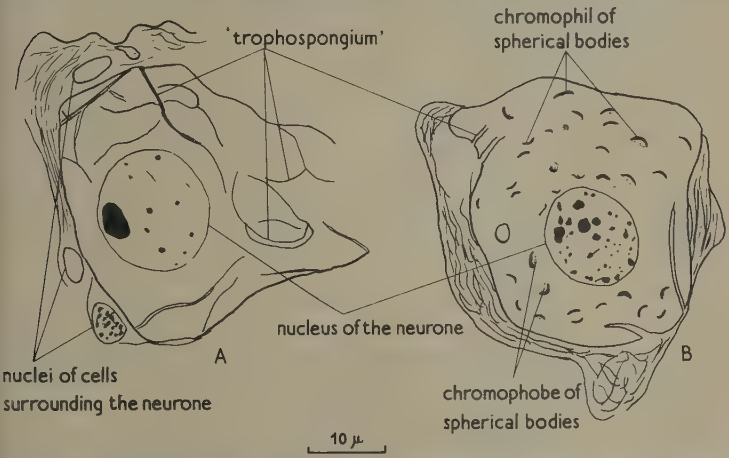
'trophospongium'
chromophil of spherical bodies
nucleus of the neurone
A
B
nuclei of cells surrounding the neurone
chromophobe of spherical bodies
10 μ

FIG. 5. Neurones of *Leander* from Helly (with postchroming) / iron haematoxylin preparations, showing the branching of 'trophospongial' structures in the cytoplasm of the neurone in A.

Lewitsky-saline / haematoxylin preparations also sometimes show spherical bodies with a thin chromophil ring or crescent covering the chromophobe interior (fig. 6). These are sometimes distorted, and then look like bright spaces. The chromophil material in such cases lies along their edges. There is no doubt that these are the same objects as are seen in 'Golgi' preparations, but they are not swollen now.

Both the kinds of objects, namely, the structures corresponding to the spherical bodies of the living cell and tape-like extensions from the cell surface into the cytoplasm, are coloured alike by xylidine red (Masson) after fixation in Helly followed by postchroming. Mallory's phosphomolybdic acid / haematoxylin stains the tape-like extensions, but their further branching is not clearly revealed.

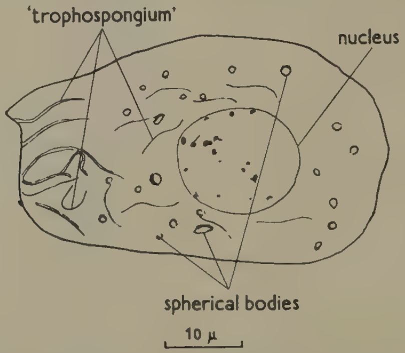

FIG. 6. Neurone of *Leander* from Lewitsky-saline / iron haematoxylin preparations.

The spherical objects do not resist paraffin embedding after fixation in Carnoy.

The mitochondria of these cells are very difficult to study in fixed preparations. The long filamentous branches of the tape-like structures are very easy to confuse with long filamentous mitochondria, but such mitochondria seem not to exist in the neurone of *Leander*. In suitable Metzner preparations minute granules, granules lying in short rows, and small rods are seen. These appear to be the mitochondria: they resemble the objects that are vitally dyed by Janus green (fig. 3). In collaboration with Dr. Meek, I have confirmed by electron microscopy that the mitochondria of these cells are mostly small granules and short rods (1959).

In neurones fixed in Zenker or Helly and stained in pyronine / methyl green (Jordan and Baker, 1955) or cresyl violet (Fernstrom, 1958) or basic fuchsine, there is an intense general staining in the cytoplasm; but there are no obvious Nissl bodies of the kind so commonly seen in the neurones of vertebrates (see Malhotra, 1959, 1960 for references). If the sections are treated with ribonuclease (Bradbury, 1956) or trichloroacetic acid (Pearse, 1954) before staining, all the basiphilia is extracted. The Nissl substance is to be regarded as uniformly dispersed.

Neurone of Astacus

The cytoplasm of the large neurones is often fixed as very coarsely granular, whereas the small and medium-sized neurones adjoining the large cells are better, but not ideally, fixed in the same section.

The cytoplasmic inclusions of the neurone of *Astacus* are similar in general to those of *Leander*. Only the points of difference are discussed here.

The spherical bodies are generally smaller than in *Leander*, varying from less than 1 μ to 1·5 μ; but in a few cells they are bigger and occasionally they are equal in size to the biggest spherical bodies in *Leander*. The osmiophil bodies vary in size in an exactly corresponding way (fig. 2, D). In contrast with the saucers and bananas seen in *Leander* after osmium impregnation techniques, the osmiophil material is usually in the form of bowls or shallow cups. The chromophobe material is more clearly seen than in *Leander*.

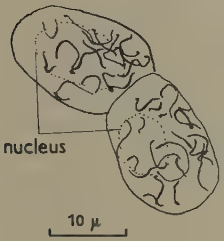

FIG. 7.

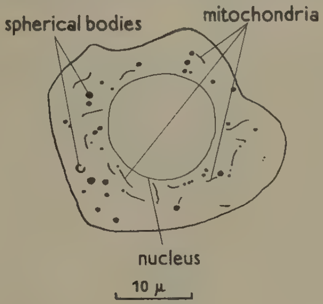

FIG. 8.

FIG. 7. Small neurones of *Astacus* from Ramón y Cajal preparations, showing the Golgi net of Monti (1915). The nuclear membrane is shown in dotted line.

FIG. 8. Neurone of *Astacus* from Altmann/Hirschler preparations. Note small rod-like objects which are probably mitochondria.

In a few small cells in Ramón y Cajal preparations long, wavy threads are seen. These are disposed in a net-like fashion (fig. 7), and resemble the loose net of Monti (1914, 1915). Similar structures are also seen in Metzner preparations, and might have been interpreted as mitochondria, if I had not studied the neurones of *Leander*. These filamentous objects resemble the branches of the tape-like structures, which spring from the surface of the cell. But such a connexion with the cell membrane has never been observed in *Astacus*, probably because the peripheral part of the cytoplasm is nearly always damaged by being pulled away from the surrounding tissue, and in the process the cell membrane is torn. The filamentous objects seem to branch much more freely than in *Leander*. I have seen similar filaments disposed in a net-like manner in the small neurones fixed in buffered osmium and studied by phase-contrast.

It is very difficult to say anything definite about the mitochondria of this cell. Vital dyeing with Janus green has not been successful and therefore there is no control. Nevertheless, the objects which are seen in Metzner or Hirschler preparations in the form of minute granules and short threads may be mitochondria (fig. 8). It is difficult to be sure, because some of these threads may be fine branches of the tape-like structures which are connected with the surface of the cell.

No birefringent material was observed in the cytoplasmic inclusions of the neurones of *Astacus*.

Histochemistry

If the ganglia of *Leander serratus* are subjected to the standard Sudan black technique of Baker (1949) after fixation in formaldehyde / calcium with postchroming (Baker, 1946), the cytoplasmic structures that are coloured correspond in size and distribution to the spherical bodies seen in the living cell. The colouring is feeble and is confined to a thin layer at the surface of these bodies. Colouring with Sudan black is very feeble after fixation in Lewitsky-saline. The acid haematein (AH) test is negative for these bodies after fixation in formaldehyde / calcium.

If Aoyama's formaldehyde-cadmium mixture is used as fixative instead of formaldehyde / calcium in the acid haematein test, the results are strikingly different. In sections of the tissue fixed in this way, postchromed as for the AH test, and coloured with Sudan black, the colour is considerably intensified at the surface of the binary spherical bodies. Acetylated Sudan black (Casselman, 1954, 1959), which is a more specific test for lipids, brings out the spherical bodies clearly. They appear sharply delimited by a thin sudanophil layer, which is usually entire (fig. 2, c), but sometimes only partial. Sometimes these objects are distorted into irregular structures. Their surface now gives also a true AH-positive reaction (positive after AH; negative after pyridine extraction). This shows the phospholipid nature of the surface of these bodies. This is in accordance with the refringent appearance seen by phase-contrast. Though Aoyama's silver impregnation method has not given satisfactory results, nevertheless if the Sudan black technique is used on material fixed in Aoyama's fluid (with postchroming), the spherical bodies are generally preserved in a life-like or almost life-like condition.

The above results suggest that the phospholipid which is localized at the surface of the spherical bodies is 'masked'; that is, it probably exists as lipoprotein complex. It does not respond to routine tests for phospholipid unless it is first liberated by the use of some 'unmasking' agent. Though tests for cytoplasmic proteins (Sakaguchi for arginine and Hg nitrite for tyrosine) are negative for the spherical bodies, it is difficult to exclude the possibility of their presence in these bodies.

The spherical bodies also react positively to the PAS test. After digestion in saliva or distase the PAS reaction is still positive, but weaker. This suggests the presence of a carbohydrate. If a ganglion is fixed in cold acetone and sections are treated by the PAS test, the spherical bodies react positively; the reaction is weaker if hot acetone in a Soxhlet apparatus is used as fixative. Colouring with acetylated Sudan black is also feebler after extraction with hot acetone than with cold acetone. The facts suggest the presence of cerebroside (Casselman and Baker, 1955), and would account in part for the positive PAS reaction.

When Cain's Nile blue test is applied to the neurones the surface of the spherical bodies is blue. This indicates the acidic nature of this part.

The spherical bodies reacted negatively to all the other histochemical tests listed in the appendix.

There are certain resemblances between the trophospongial framework and the spherical bodies in their responses to histochemical tests, but there are also the following important differences.

(1) Colouring of the trophospongial framework after Sudan black is very feeble even when an unmasking fixative is used. Whereas this framework is positive to the standard acid haematein test, the spherical bodies are negative.

(2) The trophospongial framework does not show any appreciable PAS-positive material.

(3) The spherical bodies do not resist paraffin embedding after fixation in Carnoy.

(4) The trophospongial framework does not colour in life with neutral red.

DISCUSSION

Beyond the inclusions listed earlier (p. 77), no other distinct structure can be demonstrated in the cytoplasm of the neurones of *Leander* or *Astacus* by any of the techniques used in this study.

The spherical bodies seen in the unstained living cell are coloured by neutral red. After colouring, the binary appearance of the spheres cannot be easily seen by direct microscopy. It is not clear whether neutral red colours the surface of these spheres or their interior or both. The work of Young (1953, 1956) on the neurones of cephalopods shows that the surface of the lipid droplets is more deeply coloured by neutral red. In the neurones of *Lumbricus* it is the lipid material at the surface of the 'binary spheroids' that takes up the dye (Thomas, 1954). Hirsch (1939) has also recorded that in the intestine of *Ascaris* neutral red colours the 'externum' (which is also blackened by osmium tetroxide), while in the pancreas of the white mouse (Hirsch, 1959) neutral red sometimes colours the 'externum' and sometimes the 'internum'. It is, therefore, likely that in those lipid globules that exhibit a clear differentiation into a lipid sheath and a non-lipid interior, neutral red is capable of colouring both the components. It is, however, uncertain what determines the uptake of this basic dye, which is avoided by almost everything except lipid droplets in the cytoplasm of most animal cells. It is probable that the phosphoric group of the phospholipids attracts the dye at the surface. The colouring of the internum is not explained. It may perhaps be due to the presence of acidic mucopolysaccharide, which is thought often to occur in the interior of such bodies, or to the presence of ribonucleoprotein (compare Dustin, 1947).

Experiments with neutral red on the neurones of *Leander* show that colouring by this dye does not interfere with the optically visible structure of the bodies it colours.

The objects commonly called 'Golgi apparatus' or 'dictyosomes' in the neurones of invertebrates (see p. 80 for references) are commonly represented by spherical bodies in life. During the process of fixation the spherical bodies often break open into rods and crescents (Baker, 1958; Chou and Meek, 1958). These represent the lipid surfaces of the spheres, and are the basis of reduction of osmium tetroxide or silver nitrate. This is in conformity with the findings of Young (1953, 1956), Shafiq (1953), Chou (1957 *a*, *b*), Chou and Meek (1958), and Baker (1959), who regard the 'Golgi apparatus' of the neurones of invertebrates as represented by neutral red globules in the living cell. I also arrived at this conclusion in studies of the neurones of insects and molluscs (1956, 1957). Chou and Meek (1958) have found that the lipid globules, which are often seen as crescents and rods in the electron micrographs of the neurones of *Helix aspersa* after fixation in buffered osmium, appear as circles (as in life) if calcium chloride is added to the fixative (Baker, 1958). Similar results have been obtained with the neurones of *Leander* when osmium tetroxide in sea-water is used as a fixative instead of Mann's fluid (in Mann–Kopsch). This may perhaps be due to the presence of calcium in sea-water.

In addition to the spherical bodies, there is another structure in the neurones of Crustacea which has been labelled in the past as the 'Golgi apparatus'. This is the 'trophospongium' of Holmgren (Ross, 1915, 1922; Welsh and Schallek, 1946). It originates as narrow hollow structures at the surface of the cell, which branch into fine threads, ramifying throughout the cytoplasm (Ross, 1922). Recently Wigglesworth (1959) has described very similar structures in the neurones of the hemipteran, *Rhodnius prolixus*. He regards them as branching invaginations of the surface membrane, into which the glial cytoplasm extends. Hess (1958) also observed similar invaginations in electron micrographs of the neurones of the cockroach, *Periplaneta americana*.

Monti (1915) and Migliavacca (1929) mistook the fine thread-like branches of the 'trophospongium' for mitochondria, because they are also stained by mitochondrial techniques. They thought that the Golgi network was formed by the deposition of silver on these threads. Monti and Migliavacca did not realize that these threads were parts of the 'trophospongium'.

Moreover, Monti, being a pupil of Golgi, did not believe in the existence of 'Golgi apparatus' in the form of separate elements. She considered that the short, straight or slightly sickle-shaped bodies seen by Poluszynski (1911) were artifacts of prolonged reaction. Poluszynski was in fact looking at the lipid sheaths of the spherical bodies, which had burst open into rods and crescents. He also observed a life-like or almost life-like appearance of the spherical bodies in the form of 'rings' and also of 'granules' which had blackened all through, but he regarded them as swollen threads, which, he believed, were the real form of the Golgi apparatus. Migliavacca considered that the 'rings' were a particular developmental stage of the mitochondria. If they had controlled their results with the study of the living cell and had used vital dyeing, they would not have arrived at these conclusions.

Ross (1922) felt certain that the filamentous Golgi network seen by Monti (1915) in the small and medium-sized neurones of Crustacea was quite independent of the mitochondria. Although he observed and described in detail the delicate filaments of the 'trophospongium', he thought that the Golgi network was a separate body, and did not realize that what he saw in Golgi preparations was in fact the branching threads of the 'trophospongium'. Ross, however, thought that the objects described by Poluszynski (1911) were probably components of the trophospongial framework. This does not seem to be true.

With regard to the large cells, Ross stated that no one previously had succeeded in showing a typical reticular Golgi apparatus; and he also could not do so, as these cells are very difficult to fix well and do not often show the ramification of the trophospongium.

Parat (1928) claimed that the Golgi appearances were produced by the deposition of osmium or silver on the periphery of the 'neutral red vacuoles'. This is certainly true, and there is no doubt that he was dealing with spherical bodies. Since the modern techniques of histochemistry were unknown to Parat, he could not have realized that his 'neutral red vacuoles' had a sheath of lipid material. His conclusion that the neutral red vacuoles correspond to the trophospongial system of Holmgren was purely hypothetical and unfounded on fact.

Dornesco (1934) and Lacroix (1935) claimed the existence of neutral red 'vacuoles' independent of the osmiophil and argentophil bodies ('dictyosomes'). My results do not support this. Moreover, Dornesco reported that the neutral red vacuoles in the neurones of *Potambius* run together with the passage of time. I have never observed this in the neurones of *Leander* while the cell is alive.

I have not encountered in the neurones of *Leander* or *Astacus* the small, compact and localized net described by Monti (1914, 1915) and Migliavacca (1929) in the neurones of *Astacus* and *Homarus*.

My results do not confirm the findings of Lacroix (1935) on the existence of distinct Nissl bodies in the neurones of *Astacus*. My observations show that the Nissl substance is uniformly dispersed throughout the cytoplasm in the neurones of Crustacea. This is in agreement with the observations of Ross (1922). Recently Moussa and Banhawy (1959) have also recorded that the Nissl bodies in the various developmental stages of *Schistocerca gregaria* are in the form of granules which are evenly distributed throughout the cytoplasm of the neurones of the thoracic ganglia. The evidence suggests that while the Nissl substance of the neurones of vertebrates is aggregated into lumps (Palay and Palade, 1955; Hess, 1955; Palay, 1956; Palade, 1958; Barton and Causey,

1958) that of invertebrates is more uniformly dispersed through the cytoplasm (De Robertis and Bennett, 1954; Hess, 1958). Schultz and his colleagues (1956) have made comparable observations on the sensory neurones of the spinal cord of the lamprey. They saw no Nissl bodies and in conformity with this they found no endoplasmic reticulum in electron micrographs. Chou and Meek (1958) working with the neurones of *Helix aspersa* did not encounter in their electron micrographs any aggregations of the endoplasmic reticulum suggestive of lumps of Nissl bodies.

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APPENDIX

A summary of the histochemistry of the spherical bodies and tape-like prolongations ('trophospongium') in the neurones of Leander serratus

Test	*Fixation*	*Embedding medium*	*Thickness of the sections*	*Reference*	*Spherical bodies*	*'Tropho-spongium'*	*Ground cytoplasm*
			(μ)				
Natural colour					colourless	colourless	colourless
Neutral red	living tissue				+++	O	O
Dahlia	living tissue				O	O	++
Janus green	living tissue				O	O	mitochondria are coloured
Masson or xylidine red	H + PC	P	5	Pantin, 1948	orange	orange	O
Metzner	Alt; H + PC	P	3	Metzner and Krause, 1928	+++	+++	+
Hirschler	Alt; H + PC	P	3	Hirschler, 1927	+++	+++	+
Rawitz	Flm	P	6	Baker, 1959; Przełęcka, 1959	+++	+++	+
Pyronine / methyl green	Z 3 h	P	6	Jordan and Baker, 1955	O	O	+++
P/MG after ribonuclease	Z 3 h	P	6	Bradbury, 1956	O	O	O
P/MG after trichloroacetic acid	Z 3 h	P	6	Pearse, 1954	O	O	O
Basic fuchsine or cresyl violet	Z; H	P	5	Fernstrom, 1958	O	O	+++
Sakaguchi	Z	P	10	Baker, 1947	O	O	++
Hg/nitrite	F/SW	C	20	Baker, 1956*b*	O	O	++
Metachromasy	Z	P	10	Baker (unpublished)	O	O	O
Toluidin blue after sulphation	Z 3 h	P	8	Bignardi, 1940	O	O	O
Sudan IV	F/Ca + PC	G	10	Herxheimer, 1901	O	O	O
Sudan black	F/Ca + PC; LS	G	10	Baker, 1949, 1956*a*	+	+	O
Sudan black	Aoy + PC	G	10	Baker (unpublished)	++	+	O
Acetylated Sudan black	Aoy + PC	G	10	Casselman, 1954	++	+	O
Acid haematein	F/Ca + PC	G	10	Baker, 1946	O	+	O
Acid haematein	Cr/F	G	10	Lewitsky, 1931	O	+	O
Acid haematein	Aoy + PC	G	10	Baker (unpublished)	++	+	O

Acid haematein with pyridine extraction	WB+PE	G	10	Baker, 1946	O	O	O
Nile blue	F/Ca+PC	G	10	Cain, 1947	+(very feeble)	+	O
Nile blue	Aoy+PC	G	10	Cain, 1947	+	+	O
Plasmal	fresh tissue			Cain, 1949; Pearse, 1954	O	O	O
Liebermann	F/SW	G	10	Lison, 1953	O	O	O
Performic acid / Schiff	Z 3 h	P	6	Pearse, 1954	O	O	O
Periodic acid / Schiff	Z 3 h	P	6	McManus, 1948; Pearse, 1954	++	O	O
PAS after diastase	Z 3 h	P	6	Pearse, 1954	+	O	O
PAS after saliva	Z 3 h	P	6	Pearse, 1954	+	O	O
PAS after cold acetone	C Acet	G	10	McManus, 1948	++	O	O
PAS after hot acetone	H Acet	G	10	McManus, 1948	+	O	O
Acetylated Sudan black after cold acetone	C Acet	G	10	Casselman and Baker, 1955	++	—	—
Acetylated Sudan black after hot acetone	H Acet	G	10	Casselman and Baker, 1955	+	—	—
Gomori's for alkaline phosphatase	Alc/Acet	P	8	Gomori, 1952	O	O	O

Alc/Acet = absolute alcohol and acetone in equal volumes; Alt = Altmann; Aoy+PC = Aoyama with postchroming; C = collodion; C Acet = cold acetone; Cr/F = chromium trioxide and formaldehyde; F/Ca+PC = formaldehyde/calcium with postchroming; Flm – Flemming with acetic acid; F/SW = formaldehyde in sea-water; G = gelatine; H = Helly; H Acet = hot acetone; H+PC = Helly with postchroming; LS = Lewitsky-saline; P = paraffin; WB+PE = weak Bouin+pyridine extraction; Z = Zenker; Z 3 h = Zenker (3 hours). +++ = strong reaction; ++ – moderate reaction; + = weak reaction; O = negative; — = no observation made.

Is Protoplasm ever Fluid?

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SUMMARY

The viscosity of the hyaline (granule-free) cytoplasm of the immature egg of *Spisula* (Lamellibranchia) was determined by use of the Einstein equation for Brownian movement. The value obtained was 4·3 centipoises. Thus this protoplasm has a viscosity similar to that of a concentrated protein solution. The viscosity of the entire cytoplasm, granules and all, is much higher. This is due to the great concentration of granular material. Study of the movement of the large germinal vesicle through the cytoplasm under the influence of different centrifugal forces indicates that the viscosity of the entire cytoplasm, granules and all, does not decrease as the shearing force increases. Hence, apparently, the entire cytoplasm behaves like a true Newtonian fluid—at any rate there is no evidence of thixotropy. The idea that protoplasm is typically a gel is not in accord with the evidence presented here. Additional evidence for the fluidity of protoplasm is provided by earlier information about the osmotic behaviour of cells, the formation of ice crystals in protoplasm, the rapid dispersion of aqueous solutions injected into cells, the outflow of protoplasm from cells broken in calcium-free media, and by magnetic studies.

FROM the very beginning of the study of protoplasm, biologists have been concerned with the question whether protoplasm commonly was fluid or whether it has some of the properties of a solid. With the rise of the science of colloid chemistry, this question became translated into terms of sol and gel.

Many distinguished authorities have felt it necessary to believe that in view of the complicated processes that occur in any living cell, protoplasm could not be fluid. Thus in his classical book on general biology, Oscar Hertwig (1909) was led to state: 'Wie Nägeli und viele andere Forscher, sind wir der Überzeugung, daß die komplizierten Erscheinungen des Lebensprozesses, vor allen Dingen der Vererbung, nicht aus den Eigenschaften von Flüssigkeiten oder gelösten Stoffen erklärbar sind', and he was of the opinion that the only thing that is fluid about protoplasm is the water it contains. This opinion has persisted to the present. Wald (1954) wrote: 'Lately it has become clear that very little of a living cell is truly fluid.' In 1956 Swann wrote that 'even the most fluid-seeming protoplasm is in fact a gel'. Perhaps in recent years what has been the most convincing argument in favour of a solid protoplasm is the fact that in many different types of cells a complicated reticulum has been discovered with the aid of the electron microscope.

The question whether or not protoplasm is ever fluid is an important one for various branches of physiology. So, for example, for any theory of muscular contraction it is important to know whether the mechanical changes that occur are due to a sol–gel or gel–sol transformation. So too the pharmacologist is

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interested, or should be interested, in knowing what various drugs may do to the colloidal properties of protoplasm, and in order to understand these effects it is necessary to have some notion as to what untreated protoplasm is like. Similarly, the pathologist is concerned, or should be concerned, as to how degenerative changes affect the protoplasmic colloid.

In what follows, no attempt will be made to discuss the voluminous literature concerning the fluidity or viscosity of protoplasm. Such discussion will be found in various books (Heilbrunn, 1928, 1956, 1958). All that we shall do here is to attempt an answer to the argument that protoplasm is never fluid. Actually it is hard to understand how such an argument could ever have arisen. For when Swann (1956) states that the most fluid-seeming protoplasm is in fact a gel, he can scarcely believe that the protoplasm in an amoeba or in a plant cell, such as a cell of *Nitella* or *Elodea*, is not *actually* flowing. And if it is flowing, it is by definition a sol, not a gel. And how can one speak of sol–gel change in protoplasm, as Swann does, if protoplasm is never in the sol state?

We should like to raise the following points, buttressing them by new observations whenever possible. Some of the points may be open to argument, but the sum total of the evidence seems convincing.

1. Living cells show osmotic behaviour. They swell when placed in hypotonic solutions. This, it seems to us, could only happen if the interior of the cell were fluid. Gels do not ordinarily swell in hypotonic solutions; indeed, the presence of certain salts in increasing concentration often favours the swelling of gels. In one experiment, performed recently, eggs of *Spisula solidissima* (Lamellibranchia) were placed in a solution consisting of 60 volumes of sea-water and 40 volumes of distilled water. (This is a hypotonic solution, for sea-water is isotonic for the clam eggs.) In the hypotonic solutions, the volume of the eggs increased on the average from 32,000 to 42,000 cu. μ. On the contrary, eggs treated with osmium tetroxide in sea-water and then immersed in 60 volumes of sea-water with 40 volumes of distilled water did not show any increase in volume. Indeed, their volume, if it changed at all, seems to have suffered a slight decrease—from 41,900 to 38,800 cu. μ. In the osmium tetroxide solution, the protoplasm is quickly converted from sol to gel, as can readily be shown by viscosity tests made by the centrifuge method.

Although modern cell physiologists are generally agreed that living cells show osmotic behaviour, a contrary view is held by Troschin (1958).

2. If frog muscle-fibres are cooled to -1 or $-2°$ C and are then seeded with ice crystals, masses of ice crystals form within the protoplasm (Chambers and Hale, 1932). A gel would not behave in this way, for ice crystals appear in gels only at very low temperatures.

3. When marine egg cells, for example sea-urchin eggs, are placed in solutions from which calcium has been removed by oxalate, and are then compressed until their outer membrane breaks, the protoplasm gushes out in an unbroken stream and then mixes with the surrounding medium. No one watching this unhampered flow would be likely to doubt the fluidity of the protoplasm. Similar outflows can be observed in protozoan cells. In the case

of the giant amoeba, *Chaos chaos*, the outflow occurs not only in the absence of calcium, but also in dilute solutions of heparin (Heilbrunn, Ashton, Feldherr, and Wilson, 1958). Often when amoebae are placed in dilute heparin solutions, the outflow occurs even without external pressure, and the material in an advancing pseudopod disperses into the surrounding watery medium.

4. If an isolated muscle-fibre of a frog is injected with a saline solution coloured with a vital dye, the fluid as it is injected mixes with the interior protoplasm readily. This would presumably not be the case if the protoplasm were a gel, and indeed if the protoplasm is transformed into a gel by one treatment or another, the injected fluid remains as a separate droplet.

5. If specimens of *Amoeba dubia* are made completely quiescent by placing them in dilute solutions of potassium oxalate and are then examined with a horizontal microscope, it can be seen that the small, dark granules of this amoeba fall through the cell under the influence of gravity. There is no evidence of a network, and sometimes the stratification of granular material is so clear that the amoebae look like centrifuged marine eggs. Numerous experiments of this sort were done in our laboratory by Mr. Carl Feldherr. In these experiments it is essential that amoebae be completely motionless, for the force exerted by even a slight amoeboid movement is more powerful than gravity (as can readily be calculated from the speed of the movement compared to the speed of fall under gravity). Studies made on amoebae which are not completely quiescent obviously have no meaning. In oxalate solutions the viscosity of the protoplasm in the interior of an amoeba is increased, as has been shown both by the centrifuge method (Heilbrunn and Daugherty, 1933; Alsup, 1942) and also by the magnetic method to be described in the next section. In the case of marine eggs, it is of course not necessary to immobilize the cells. In the case of sea-urchin eggs and also eggs of the worm *Chaetopterus*, we have frequently observed granules falling through the cytoplasm merely under the influence of gravity; similar observations have been made by Howard (1932).

6. Correct magnetic studies of the protoplasmic colloid indicate fluidity. Years ago, Heilbronn (1922) introduced iron wires into large masses of slime mold protoplasm and then twisted them with an electro-magnet. In this way he obtained low values for the viscosity of the interior protoplasm, values much like those obtained with the centrifuge methods and the Brownian movement method for other types of protoplasm. Only in small slime molds, when the wires impinged on the outer cortex, was there any evidence of thixotropy: otherwise the protoplasm behaved like a true Newtonian fluid. More recently Crick and Hughes (1950) studied the effect of magnetic forces on iron particles taken up by fibroblast cells, and obtained some evidence of elasticity of the protoplasm. They recognized the fact that the method was not properly applicable if the magnetic particle was near a limiting membrane or if it was in a vacuole. Fibroblasts are very flat cells, and it is hard to imagine a particle being anything but close to a limiting membrane. In the one photograph that Crick and Hughes show, the iron particle is directly in contact with

the nuclear membrane. Also it seems clear that particles taken up by amoeboid cells are almost certainly in vacuoles.

The most interesting study is that of Ashton (1957), thus far reported only in preliminary fashion. He found that iron particles within the flowing protoplasm of a giant amoeba (*Chaos chaos*), when twisted by magnetic forces, showed no evidence of elasticity in the medium surrounding them. Moreover, movement of an iron particle with a magnet calibrated so as to give a force exactly equal to gravity at a distance of 1 cm gave values which could be used to calculate the viscosity of the flowing protoplasm. This calculation was made by the aid of Stokes's law. The values thus far obtained are essentially in accord with earlier values for amoeboid protoplasm, obtained both by the centrifuge method (Heilbrunn, 1929) and by the Brownian movement method (Pekarek, 1930). These values clearly show the fluidity of the flowing protoplasm of an amoeba, so that now there is agreement in the results obtained by three different methods.

Ashton has repeatedly attempted to shoot iron particles into sea-urchin eggs by placing a strong magnet under a mass of eggs packed rather closely together as a result of centrifugal treatment. Usually the eggs are completely smashed by this procedure, but in one case an apparently uninjured egg was caused to contain a tiny iron rodlet. This could readily be twisted by a magnetic field, almost as readily as the iron rodlets outside the egg. Then, on removing the magnet, both the rodlet inside the egg and those outside swung around so as to point to the magnetic north like tiny compasses. Anyone observing this experiment, as we did, could not fail to be impressed by the fluidity of the protoplasm. It is a much more significant experiment than the oft-quoted observation of Seifriz (1924). The latter never claimed to have got a magnetic particle into a cell interior, but only in one case into the cortex of a sand-dollar egg. Even this is doubtful according to a statement made to one of us by Robert Chambers, under whose direction the work was done. According to Chambers the nickel ball that Seifriz used only indented the protoplasm, and under the influence of the magnetic field the whole egg rotated. Actually it would scarcely be possible to push a large nickel ball (16 μ in diameter) into a cell merely by poking it with a microneedle. And yet this dubious experiment continues to be quoted, most recently by Giese (1957), who wrongly attributes the observation to Frey-Wyssling (Giese, 1958).

7. Perhaps the most impressive support for the belief that the protoplasm of cells is typically a solid mass comes from many recent studies with the electron microscope. Valuable as this instrument is, it can be used only on dead material. The cells have been treated with drastic fixatives and have then had all water removed from them. The recent work is reminiscent of the older studies of cytologists in the latter half of the nineteenth century. At that time Frommann and Heitzmann among the zoologists and Strasburger and Schmitz among the botanists claimed that the essential nature of protoplasm derived from the fact that it was a network. This older work is discussed in some detail by Berthold (1886) and by Bütschli (1892), and numerous references can be

found there. Various authors, including Hardy (1899), Fischer (1899), and Mann (1902), studied the effect of common fixatives such as osmium tetroxide, formaldehyde, chromic acid, dichromate, &c., on protein solutions and were able to show that these fixatives caused a precipitation which might take the form of granules, filaments, or networks. Moreover, the older observations which indicated the presence of a network in living cells were sharply criticized by Bütschli, who pointed out that the vacuolization which so often accompanies death or degenerative changes was often erroneously described as representing a protoplasmic network. A similar mistake has apparently been made recently. Thus Fawcett and Ito (1958), in their attempt to show the presence of an endoplasmic reticulum in living cells, seem to have fallen into this error in their study of cells removed from the testes of guinea pigs. In cells that the authors state were 'gradually succumbing to unfavorable environmental conditions', they described various structures, some of which are certainly typical vacuoles such as have repeatedly been described in dying or injured protoplasm. They believe that the appearance of what they consider to be a reticulum is not to be regarded 'as a terminal event in moribund cells', for in these obviously dying cells the mitochondria stay intact. This seems to be a rather weak argument, for even in smashed-up cells, mitochondria may stay intact. Moreover, in the cells that Fawcett and Ito tear out of the testis, cells which are presumably already somewhat injured by the isolation process, there is no observable endoplasmic reticulum.

8. If an endoplasmic reticulum, or some structure similar to it, were really present in a living cell, then the protoplasm of such a cell would presumably not behave as a fluid. Because lamellar structures comparable to an endoplasmic reticulum have been described in the immature egg of the surf-clam, *Spisula solidissima* (Rebhun, 1956), it was thought wise to attempt a physical study of the living egg to see if support could be found for Rebhun's studies with the electron microscope. There was also an additional reason for studying the immature *Spisula* egg. As with other immature eggs with large germinal vesicle, the cytoplasmic granules in the cytoplasm do not move readily under the influence of centrifugal force, far less readily than such granules move in the mature eggs of the sea urchin *Arbacia*, the worm *Chaetopterus*, or indeed in the mature egg of *Spisula* itself. Indeed it is generally held that the protoplasm of immature eggs is much more viscous than it is after the germinal vesicle has broken down. Because of the difficulty encountered in getting the granules to move through the cytoplasm of immature *Spisula* eggs, Allen (1953) was led to conclude that the cytoplasm was a gel, and he found support for this opinion in the fact that, after centrifuging, a ring around the nucleus is negatively birefringent.

The work on *Spisula* protoplasm was done at the Marine Biological Laboratory in Woods Hole. Details of our work on *Spisula*, as well as similar work on other marine eggs, will be presented in a paper to be published later. The main facts are these. When *Spisula* eggs are centrifuged so that the heavy granules of the cytoplasm are moved to the centrifugal end of the cell, the rate of return of these granules can be measured. Then by applying the Einstein equation for Brownian movement, the viscosity of the hyaline (non-granular) cytoplasm can be measured. The Einstein equation is

$$D_x^2 = \frac{R}{N} \cdot \frac{Tt}{3\pi\eta a},$$

in which D_x is the displacement of a particle along one axis in the time t, and at the absolute temperature T, R is the gas constant, N the Avogadro number, η the viscosity, and a the radius of the moving particle. In order to apply the Einstein equation, it is only necessary to know D_x, t, and a. It is not difficult to determine D_x, although no great accuracy is possible. Measurement of the diameter of the heavy granules gave a value of approximately 0·8 μ. This measurement is also somewhat uncertain, for the granules are never completely at rest while the measurement is being made. We centrifuged the eggs for 4 min with a force 9,000 times gravity. In eggs taken from small clams, this was sufficient to move the heavy granules as far as they would go toward the centrifugal end of the egg. The fatty granules moved in the opposite direction; usually some of them formed a ring around the large nucleus, and this is doubtless why Allen found this region to be negatively birefringent.

TABLE I

The viscosity of the hyaline protoplasm of immature Spisula *eggs*

D_x	t	$\eta \times 100$
(μ)	(sec)	(centipoises)
10	360	4
15	300	1·5
10	360	4
15	518	2·5
8	480	8
10	480	5
8	595	10
10	180	2
6	149	4·5
6·5	152	4
13	225	1·5
9	344	4·6
10·5	366	3·7
6	174	5·3
		Mean 4·3

We measured the time it took for some of the heavy granules to move a given distance (D_x), and thus we were able to calculate the viscosity. Table I shows the results of 14 determinations. The average value for the viscosity was found to be 4·3 centipoises. This is the type of value that one might expect for a concentrated protein solution. After the eggs have been fertilized, the large nucleus breaks down and releases its fluid into the cytoplasm. Then as the concentration of granular material becomes much less, the granules move much more readily when the eggs are centrifuged. And yet, surprisingly enough, the viscosity of the hyaline protoplasm through which they move remains approximately the same.

The low value for the viscosity of the hyaline protoplasm of the *Spisula* egg is surely an indication of the fluidity of this non-granular protoplasm. It might perhaps be objected that the Einstein equation is not very accurate; and indeed it is not, for in its derivation Einstein took no account of the electrical charges on the moving particles. Although earlier tests of the equation gave results consistent with it, some more recent studies have shown that the observed values for D_x may be less than the calculated values. For example, Bender and Mouquin (1952) found observed values only one-third to two-thirds as great as the values obtained from the equation. But any divergence of this sort would only lower the values obtained in our studies and would thus indicate that the protoplasm was even more fluid than we found it to be. On the other hand, when granules are centrifuged to the heavy end of the egg, their Brownian movement is not perfectly random, at least at the start, for their movement in the centrifugal direction would tend to be blocked. This would tend to increase the value for D_x in the centripetal direction. Fortunately, these two sources of error would tend to balance each other.

Also, it might be argued that the centrifugal force used to move the granules destroys a reticulum originally present in the egg cytoplasm. This is possible, and yet the *Spisula* egg is unusually resistant to centrifugal force, and it can develop normally even after violent centrifugal treatment. Certainly the centrifuge does not cause anything like the damage that fixation and desiccation do. It is also conceivable that the cytoplasmic reticulum has wide meshes and that the small granules sift through it almost as if it were not there. Moreover, in Rebhun's studies, the lamellae which he described occupy only a small volume of the cytoplasm. However, Palade, one of the leading authorities on the endoplasmic reticulum, believes that 'it is present in all animal cells' (Palade, 1956), and he notes that 'it appears that the endoplasmic reticulum is a continuous network of membrane bound cavities permeating the entire cytoplasm from the cell membrane to the nucleus'.

It may be difficult to show with any certainty that no reticulum of any sort exists in the cytoplasm, but if there is a reticulum it must be a weak structure indeed, one without much influence on the physical nature of the cytoplasm. For if one determines the rate of movement of the large nucleus (germinal vesicle) under the influence of centrifugal force and uses this rate of movement as a test of the viscosity of the entire protoplasmic suspension, granules and all, then it can be shown that the relative viscosity determined in this fashion remains the same whether a force 9,000 times gravity is employed, or a force only one-fourth as great. Thus within this range of shearing forces, the protoplasmic suspension behaves like a true Newtonian fluid. Accordingly it seems safe to conclude that if any reticular structures are present, they can be of little importance in influencing the colloidal state of the main mass of the protoplasm.

If our observations are correct, then it is clear that the cytoplasm of the immature *Spisula* egg owes its high viscosity to the high concentration of granules within it. Such a high concentration would undoubtedly lead to high values for the viscosity of the entire protoplasmic suspension (for a discussion of the viscosity of concentrated suspensions, see Heilbrunn, 1958). And yet the hyaline, non-granular protoplasm is quite fluid. Perhaps in all cells with a high concentration of granular or filamentous material, the viscosity of the hyaline protoplasm or ground substance may be low. This is apparently true for the protoplasm of *Paramecium*, as indicated by the work of Larsen, which was discussed in some detail by Heilbrunn (1956). In the near future we hope to investigate the physical properties of the cytoplasm of other types of cells in which the cytoplasm is clogged with granular material.

In conclusion, the important question is really this. If we are to understand the physical properties of protoplasm, are we to depend on the information gained from coagulated, thoroughly desiccated cells, which have lost every vestige of vital activity, or should we not lay more stress on what can be learned from physical studies of cells which are alive and uninjured? The methods we use for a study of protoplasm may not be perfect, but until better methods are discovered, it is to these methods that we must look for proper information. Both centrifugal measurements and measurements of Brownian movement have shown that in various types of cells, both plant and animal, the main mass of the cytoplasm has a low viscosity. We know of no proper measurement for any uninjured cell that has given information to the contrary, and therefore we deny that 'even the most fluid-seeming protoplasm is in fact a gel'.

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